Molecular Psychiatry (2002) 7, 718–725

www.nature.com/mp

ORIGINAL RESEARCH ARTICLE

Serotonergic system and attention deficit hyperactivity disorder (ADHD): a potential susceptibility locus at the 5-HT_{1B} receptor gene in 273 nuclear families from a multi-centre sample

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Attention deficit hyperactivity disorder (ADHD) is a highly heritable and heterogeneous disorder, which usually becomes apparent during the first few years of childhood. Imbalance in dopamine neurotransmission has been suggested as a factor predisposing to ADHD. However, evidence has suggested an interaction between dopamine and serotonin systems in the pathophysiology of the disorder. Studies using selective agonists of the different 5-HT receptors microinjected into selected brain structures have shown a positive modulating effect on the functional activities of the mesotelencephalic dopaminergic system. This suggests that some of the genetic predisposition to ADHD might be due to DNA variation at serotonin system genes. In this study, we investigated polymorphisms in HTR_{1B} and HTR_{2A} (which encode the serotonin receptors 5-HT_{1B} and 5-HT_{2A} respectively) in a European ADHD sample. Using haplotype based haplotype relative risk (HHRR) and transmission disequilibrium test (TDT) analyses, we observed significant preferential transmission of the allele 861G of the HTR_{1B} in the total sample (for HHRR; $\chi^2 = 7.4$, $P = 0.0065$ and TDT; ($\chi^2 = 6.4$, $P = 0.014$). Analysis of HTR_{2A} failed to reveal evidence of association or linkage between the His452Tyr polymorphism and ADHD in the total sample. However, a significantly increased transmission of the allele 452His was observed in the Irish sample alone ($\chi^2 = 4.9$, $P = 0.026$). These preliminary data suggest an important role for the serotonin system in the development of ADHD. Further studies, preferentially including different ethnic groups are required to substantiate these findings.
Molecular Psychiatry (2002) **7,** 718–725. doi:10.1038/sj.mp.4001048

Keywords: 5-HTR_{1B}; 5-HTR_{2A}; attention deficit hyperactivity disorder (ADHD); haplotype based haplotype relative risk (HHRR); transmission disequilibrium test (TDT)

Introduction

Attention deficit hyperactivity disorder (ADHD) is a highly heritable condition, which usually becomes apparent during the first few years of childhood. The central behavioural symptoms of inattention, impulsiveness and hyperactivity often co-occur with other behavioural conditions particularly conduct and oppositional defiant disorders. These disabilities lead to significant repercussions such as aggression and other anti-social behaviour, academic underachievement, peer rejection and family dysfunction.[1] Several lines of evidence have implicated dopaminergic system genes in the aetiology of neuropsychiatric and behavioural conditions such as schizophrenia, depression, anxiety and alcoholism.[2] Imbalance in dopamine neurotransmission has also been suggested as a factor predisposing to ADHD. Brain imaging studies have shown abnormalities in the frontal lobe and subcortical structures such as the basal ganglia, regions known to be rich in dopamine neurotransmission and important in the control of attention and response to organisation.[3–5] Evidence of association between the dopamine transporter gene (DAT1) and ADHD was first described by Cook *et al*[6] and subsequently replicated by Gill *et al*,[7] Waldman *et al*,[8] Curran *et al*[9] and extended by Daly *et al*.[10] However other studies have failed to replicate this finding.[11,12] More recently, Daly *et al*[10] identified two additional potential susceptibility loci at dopamine D5

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Received 7 August 2001; revised 31 October 2001; accepted 14 November 2001

receptor gene (DRD5) (148-bp allele of a dinucleotide repeat located 18.5 kb to the coding sequence) and at dopamine-β-hydroxylase (DBH) (allele 2, Taq I polymorphism at intron 5 of the gene). Several reports of association between the 7-repeat allele in exon 3 of dopamine D4 receptor gene (DRD4) and ADHD have also been published.[13]

Pharmacological evidence has also supported the dopaminergic hypothesis for ADHD. Methylphenidate and other psychostimulant medications (dexamphetamine and pemoline), are known to inhibit the dopamine transporter[14] and to ameliorate the symptoms of ADHD.[15–19] In addition, dopamine transporter knockout (DAT-KO) mice were found to produce a phenotype that has similarities to human ADHD.[20]

Although spontaneous locomotor activity was attenuated by dopamine in DAT1-deficient mice,[21] dopamine is not the only factor that contributes to the hyperactivity and learning difficulties in these animals. Administration of methylphenidate, whose main mode of action is thought to be inhibition of the dopamine transporter, reverses the overactivity observed in DAT-KO. Furthermore, fluoxetine (a selective serotonin reuptake inhibitor) also attenuates the activity of the DAT-KO mice, whereas it had no effect on wild type animals. This action was mediated by increased extracellular concentration of serotonin (5-HT) due to blockade of the serotonin transporter.[21] In addition, when the DAT1 knockout mice were treated with 5-hydroxytryptophan or with the dietary 5-HT precursor (L-tryptophan), hyperlocomotion was profoundly reduced.[21] This occurred in the absence of any change in dopamine concentration suggesting that the serotonergic system may be involved in the pathogenesis of ADHD.

Studies using selective agonists of the different 5-HT receptors, microinjected into selected brain structures have shown a positive modulating effect of these serotonergic systems on the functional activities of the mesotelencephalic dopaminergic system. 5-HT_{1B} is an autoreceptor, which is found on presynaptic serotonergic neurons and functions to modulate the release of 5-HT. The receptor is also expressed in areas known to be involved in motor control such as the striatum, frontal cortex, medulla, hippocampus and pituitary.[22,23] The 5-HT_{1B} receptor has been implicated in locomotor behaviour. Pharmacological studies using the 5-HT_{1B} agonist RU24969 suggested that the activation of 5-HT_{1B} receptors in mice leads to increased anxiety and locomotion in these animals. The hyperlocomotion effect of this agonist was absent in the mouse lacking the 5-HT_{1B}, indicating that this agonist effect is mediated by this receptor.

The 5-HT_{2A} receptor has also recently been implicated in ADHD by linkage and association studies using a 452Tyr polymorphism in the HTR_{2A}.[24] HTR_{2A} maps to 13q14–q21 and is known to encode a protein involved in signal transduction mediated via phosphoinositol hydrolysis and intracellular Ca^{2+} mobilization. Furthermore, the hyperlocomotion induced by the non-competitive NMDA antagonist (MK-801) in mice was attenuated by the nonselective 5-HT_{2A} antagonist ritanserine and by the 5-HT_{2A} selective antagonist MDL100907.[25] Striatal administration of serotonergic agonists causes inhibition of striatal neuronal firing possibly by a decrease in synaptic dopamine. This effect is thought to be mediated by the serotonin receptor 5-HT_{2A} and may result in the decreased release or decreased synthesis of dopamine in the neuronal projections.

Based upon the above data, mutations or polymorphisms at either HTR_{1B} or HTR_{2A} which affect the structure or the expression of the these receptors have been postulated to contribute to the development of ADHD. In this investigation, we studied polymorphisms at the HTR_{1B} and HTR_{2A} gene loci in a sample of families ascertained at three centres in the UK and in the Republic of Ireland.

Materials and methods

ADHD cases were recruited from child psychiatric clinics and schools from three centres in the UK and one in Ireland. Details of the clinical assessment procedure, assignment of diagnosis and the clinical description of each sample have been published previously in the Irish by Daly *et al*;[10] Welsh /Manchester in Holmes *et al*,[12] London in Mill *et al*[26] and Birmingham in Kent *et al*.[27] Briefly, for the three UK centres, consensus diagnoses were made according to DSM-IV criteria for ADHD using the Child and Adolescence Psychiatric Assessment (CAPA),[28] a semi-structured instrument.

DNA amplification

HTR_{1B}

PCR amplification of the 548-bp region containing the HincII polymorphism was achieved as described by Lappalainen *et al*[29] using the following pair of primers: upstream, 5′ GAA ACA GAC GCC CAA CAG GAC 3′ and downstream 5′ CCA GAA ACC GCG AAA GAA GAT 3′. Cycling was for 30 rounds consisting of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. A first denaturing step at 95°C for 3.30 min and last extension step at 72°C for 7 min were also added. Depending on the quality of the amplification, approximately 10 μl PCR product was digested with 3 units of HincII as recommended by the manufacturers and separated on 2.5% agarose gels. The presence of the HTR861G allele yielded two fragments of 452 and 96 bp and the presence of HTR861C gave three fragments of 310, 142 and 96 bp.

HTR_{2A} His452Tyr

This polymorphism was amplified as described by Ozaki[30] with forward primer 5′ CAC ACA GCT CAC CTT TTC ATT CA 3′ and reverse primer 5′AGT CTA GCC AAC TTC AAA TGG 3′ to give a 155-bp product. PCR cycling consisted of an initial denaturation of 3.3 min at 95°C, followed by 30 cycles of a 30 s denaturation at 95°C, 30 s annealing at 60°C, and a 30 s extension at 72°C, and a final extension cycle at 72°C for 5

min. The 155-bp amplified fragment of HTR_{2A} His452-Tyr (7 μl) was restricted with 1 U of BsmI at 65°C for 4 h. The digests were electrophoresed on a 10% nondenaturing polyacrylamide gel at 120 V for 2.5–3 h. The HTR_{2A}/His452 allele gave two fragments of 95 and 60 bp, while the HTR_{2A}/ 452Tyr allele yielded a single uncut fragment.

Statistics

In this study we have used the haplotype based haplotype relative risk (HHRR) and transmission disequilibrium test (TDT) designs to avoid any potential population stratification. In this method, the non-transmitted parental alleles are used as 'controls' for evaluating allele transmission in the case of HHRR. In contrast, the TDT is a test of linkage and association which only uses alleles transmitted from heterozygous parents. The χ^2 test was used to examine the significance of the resulting tables.

Results

Allele frequencies for both markers did not show any significant deviation from that expected according to Hardy–Weinberg disequilibrium. Haplotype based haplotype relative risk analysis performed on the transmitted and non transmitted alleles for the marker HTR_{1B} is presented in Table 1. A significant preferential transmission of allele 861G was observed in the total sample ($\chi^2 = 7.4$, $P = 0.0065$). This was also the case for the Irish sample alone, which shows the most significant preferential transmission of this allele to ADHD cases ($\chi^2 = 9.74$, $P = 0.0018$). Increased transmission of the same allele was also seen in the samples from Wales/Manchester and Birmingham but this did not reach statistical significance in either case. However, in the sample from London, the allele 861G was less frequently transmitted to ADHD cases compared to 861C. Transmission disequilibrium test (TDT) was performed on the total sample (193 heterozygous parents) and the data are presented in Table 2. The result of the TDT analysis confirmed the previous analysis indicating the presence of linkage as well as association between the HTR_{1B} and ADHD ($\chi^2 = 6.4$, $P = 0.014$). Once again, the Irish sample exhibited the highest level of significance compared to the remainder of the sample. Similarly, the Cardiff and Birmingham samples showed a trend in the same direction. In contrast, the London sample showed a trend in the opposite direction.

In addition, HHRR and TDT analyses of transmission by parent of origin were conducted. The increased transmission observed in the total sample was largely due to the transmission of allele 861G of paternal origin to ADHD cases (Tables 3 and 4). Surprisingly, the opposite trend of transmission observed in the sample from the London group was reversed and excess transmission of the allele 861G was observed though this was not significant (Tables 3 and 4). Transmission from maternal origin to ADHD cases was not significant in the total sample (Tables 5 and 6) but was statistically significant in the Irish sample alone.

HHRR analysis of the HTR_{2A}/His452Tyr did not show any preferential transmission in the total sample (Table 7). However, considering each sample individually, we observed an increased preferential transmission of the 452His allele in the Irish sample alone ($\chi^2 = 4.9$, $P = 0.026$). This has also been confirmed by TDT analysis (Table 8) suggesting the presence of linkage as well as association between this marker and ADHD ($\chi^2 = 4.5$, $P = 0.052$) in the Irish population. An inconsistent pattern of transmissions was observed with regard to other groups; while the Birmingham sample showed a similar trend (preferential transmission of 452His allele to ADHD cases) to that of the Irish sample, Cardiff/Manchester and London samples showed the opposite trend (Tables 7 and 8).

Discussion

In this study, we observed a significant preferential transmission of allele 861G of the HTR_{1B} gene to ADHD in a European sample of 273 nuclear families. HHRR ($\chi^2 = 7.4$, $P = 0.0065$), and TDT ($\chi^2 = 6.4$, $P = 0.014$)

Table 1 HHRR analysis of HTR_{1B}/G861C in 273 ADHD nuclear families

ADHD population	*Allele*	*T*	*NT*	χ^2	*P*	*RR (95% CI)*
Ireland ($n = 80$)	861G	118	94	9.74	0.0018	1.57 (1.15–2.16)
	861C	29	53			
Wales/Manchester	861G	128	114	3.04	0.08	1.26 (0.96–1.65)
($n = 90$)	861C	37	51			
England/Birmingham	861G	63	59	0.93	0.33	1.29 (0.73–2.27)
($n = 48$)	861C	8	12			
England/London	861G	78	83	0.59	0.44	0.89 (0.67–1.19)
($n = 55$)	861C	31	26			
Total	861G	387	350	7.4	0.0065	1.24 (1.05–1.45)
	861C	105	142			

T = transmitted, NT = not transmitted, RR = relative risk.

Table 2 TDT analysis of HTR_{1B}/G861C in 193 heterozygous parents

ADHD population	*Allele*	*T*	*NT*	χ^2	*P*	*OR*
Ireland	861G	44	20	9.0	0.0037	2.2
	861C	20	44			
Wales/Manchester	861G	35	23	2.5	0.14	1.52
	861C	23	35			
England/Birmingham	861G	19	15	0.5	0.60	1.27
	861C	15	19			
England/London	861G	16	21			
	861C	21	16	0.68	0.51	0.76
Total	861G	114	79	6.4	0.014	1.44
	861C	79	115			

T = transmitted, NT = not transmitted, TDT = transmission disequilibrium test.

Table 3 HHRR analysis of HTR_{1B}/G861C transmission of paternal origin to ADHD cases

ADHD population	*Allele*	*T*	*NT*	χ^2	*P*	*RR (95% CI)*
Ireland	861G	49	37	6.47	0.01	1.9 (1.07–8.3)
	861C	9	21			
Wales/Manchester	861G	55	46	2.78	0.095	1.4 (0.91–2.13)
	861C	16	25			
England/Birmingham	861G	24	22	0.37	0.54	1.22 (0.63–2.37)
	861C	6	8			
England/London	861G	39	37	0.23	0.62	1.13 (0.68–1.88)
	861C	10	12			
Total	861G	167	142	7.85	0.005	1.41 (1.09–1.83)
	861C	41	66			

T = transmitted, NT = not transmitted, RR = relative risk.

Table 4 TDT analysis of HTR_{1B}/G861C transmission of paternal origin to ADHD cases

ADHD population	*Allele*	*T*	*NT*	χ^2	*P*	*OR*
Ireland	861G	16	6	4.5	0.052	2.67
	861C	6	16			
Wales/Manchester	861G	15	8	2.1	0.21	1.88
	861C	8	15			
England/Birmingham	861G	7	5	0.33	0.78	1.40
	861C	5	7			
England/London	861G	9	7	0.25	0.80	1.29
	861C	7	9			
Total	861G	47	26	6.04	0.018	1.80
	861C	26	47			

T = transmitted, NT = not transmitted, TDT = transmission disequilibrium test.

analyses both demonstrated the presence of association as well as linkage between this marker and ADHD in the total sample. Analysing the combined sample by population sample, the evidence for association and linkage was strongest in the Irish sample; HHRR (χ^2 = 9.74, P = 0.0018), TDT (χ^2 = 9.0, P = 0.0037). In contrast to HTR_{1B}, preferential transmission of the HTR_{2A}/452His allele was observed only in the Irish sample.

Table 5 HHRR analysis of HTR_{1B}/G861C transmission from maternal origin to ADHD cases

ADHD population	*Allele*	*T*	*NT*	χ^2	*P*	*RR (95% CI)*
Ireland	861G	62	47	8.49	0.0035	1.99 (1.15–3.45)
	861C	10	25			
Wales/Manchester	861G	68	60	2.02	0.154	1.31 (0.78–3.59)
	861C	17	25			
England/Birmingham	861G	35	33	0.25	0.51	1.14 (0.67–1.96)
	861C	9	11			
England/London	861G	34	41			
	861C	16	9	2.51	0.10	1.41 (0.96–2.07)
Total	861G	199	181	3.51	0.061	1.23 (0.96–2.28)
	861C	52	70			

T = transmitted, NT = not transmitted, RR = relative risk.

Table 6 TDT analysis of HTR_{1B}/G861C transmission from maternal origin to ADHD cases

ADHD population	*Allele*	*T*	*NT*	χ^2	*P*	*OR*
Ireland	861G	19	6	6.76	0.014	2.67
	861C	6	19			
Wales/Manchester	861G	16	9	1.96	0.087	1.78
	861C	9	16			
England/Birmingham	861G	9	7	0.25	0.8	1.29
	861C	7	9			
England/London	861G	3	11			
	861C	11	3	5.79	0.057	3.67
Total	861G	47	33	2.45	0.14	1.42
	861C	33	47			

T = transmitted, NT = not transmitted, TDT = transmission disequilibrium test.

Table 7 HHRR analysis of HTR_{2A}/His452Tyr in 274 ADHD nuclear families

ADHD population	*Allele*	*T*	*NT*	χ^2	*P*	*RR (95% CI)*
Ireland (n = 82)	452Tyr	6	16			
	452His	142	132	4.91	0.026	1.9 (0.95–3.80)
Wales/Manchester	452Tyr	21	15			
(n = 89)	452His	140	146	1.13	0.29	1.19 (0.88–1.61)
England/Birmingham	452Tyr	5	9			
(n = 48)	452His	75	71	1.25	0.26	1.84 (0.70–2.96)
England/London	452Tyr	14	8			
(n = 55)	452His	92	98	1.83	0.17	0.76 (0.54–1.08)
Total	452Tyr	46	48			
	452His	449	447	0.05	0.82	0.98 (0.79–1.21)

T = transmitted, NT = not transmitted, RR = relative risk.

Table 8 TDT analysis of HTR_{2A}/His452Tyr in 87 heterozygous parents

ADHD population	*Allele*	*T*	*NT*	χ^2	*P*	*OR*
Ireland	452Tyr	6	16			
	452His	16	6	4.5	0.052	2.76
Wales/Manchester	452Tyr	18	11			
	452His	11	18	1.68	0.26	0.61
England/Birmingham	452Tyr	5	9			
	452His	9	5	1.14	0.42	1.8
England/London	452Tyr	15	7			
	452His	7	15	2.9	0.13	2.14
Total	452Tyr	44	43	0.01	1.0	0.98
	452His	43	44			

T = transmitted, NT = not transmitted, TDT = transmission disequilibrium test.

Taken together, clinical[31] as well as molecular[10] studies indicate that ADHD is a polygenic or oligogenic disorder and that many genes (each of minor effect) are likely to be involved in the development of ADHD. Thus, the effect sizes at individual loci are likely to be small (relative risk <2), and large samples will be required to both detect and confirm linkage or association. This multi-centre study involves 273 ADHD proband–parent trios, the largest reported to date. This sample has 80% power to detect a locus contributing to ADHD with a relative risk of 3 or greater at a genome wide significance of $P<0.05$.[32] However, this analysis assumes no enhanced prior probability at any particular locus.

A substantial body of evidence has supported the involvement of the serotonergic system in the development of ADHD. Mutant mice generated by homologous recombination lacking the 5-HT_{1B} are characterized by aggressive behaviour and are found to be more impulsive than the wild type.[33] No other phenotypic or behavioural differences were observed compared to wild type animals. However, when the 5-HT_{1B} agonist RU24969 was administered to the wild-type mice, locomotor activity was enhanced but the agonist had no effect on the 5-HT_{1B}-KO mice. This suggested that the hyperlocomotor effect of the agonist RU24969 is mediated by the action of the 5-HT_{1B}. More recently, Heisler and Tecott[34] observed that when mice bearing a targeted mutation of the HT_{2C} receptor gene were treated with the non-specific serotonin receptor agonist m-chlorophenylpiperazine (mCPP), this produced hyperactivity in these animals. However, when the HT_{2C} mutant mice were pre-treated with a 5-HT_{1B} antagonist GR127935, the hyperlocomotion effect of mCPP was blocked. This compound normally suppresses locomotion in the mouse. This suggests that the apparently paradoxical hyperlocomotion effect of mCPP on the mutant 5-HT_{2C} mouse is mediated by the action of 5-HT_{1B}. When both receptor subtypes are activated by mCPP, the locomotor suppression of 5-HT_{2C} stimulation predominates. However, when the 5-HT_{2C} is removed, the 5-HT_{1B} receptor-stimulating properties of mCPP are unopposed and so lead to hyperactivity.[34] This evidence indicates that the hyperlocomotion/hyperactivity is mediated by 5-HT_{1B}.

In support of our findings, Quist *et al* also report preferential transmission of 5-HTR_{1B}/861G in 115 Canadian families. Pooling the samples in a TDT test (the present multi-centre sample plus the Canadian sample), the moderate evidence of association and linkage obtained from the European sample ($\chi^2 = 6.4$, $P = 0.014$) is strongly enhanced ($\chi^2 = 9.3$, $P = 0.0028$), indicating the possible involvement of this locus in predisposing to ADHD. It is unlikely that the 5-HTR_{1B}/G861C polymorphism would affect the net charge or the three-dimensional structure of protein since it does not result in amino acid change. It is therefore likely that another functional variant within the coding or regulatory sequence which is in linkage disequilibrium with G861C might influence the development of ADHD. A deletion involving the region 6q13–15 (where HTR_{1B} maps) was reported to cause abnormal brain development, congenital abnormalities and delayed development.[35]

A possible parent of origin effect in the development of psychiatric and complex disorders has not been fully explored, but the preferential transmission of the HTR_{1B} G861 from fathers to their ADHD offspring in the current investigation may reflect the involvement of genomic imprinting. Evidence of imprinting has been reported on chromosome 6q27, a locus which contributes to the development of IDDM8.[36] Imprinted genes tend to cluster at certain chromosomal regions but the HTR_{1B} gene maps to 6q13.[22] However, it is possible that another imprinted region may exist at or near the HTR_{1B} gene. Finally, these explanations are hypothetical speculations and differences in the HT_{1B} gene expression between paternal and maternal alleles at the G861C locus would be required to firmly implicate genomic imprinting in the aetiology of ADHD.

The HTR_{2A}/His452Tyr polymorphism maps to the C terminal end of the mature 5-HT_{2A} protein. Preliminary evidence suggested that the 452Tyr form of the protein may associate with desensitization of the 5-HT_{2A}.[37] The

possible differences in the function of His452Tyr may influence the balance of the serotonergic transmission and consequently contribute to the development of psychiatric disorders. Evidence of association between HTR_{2A}/ 452Tyr and ADHD was reported by Quist *et al*.[24] The multi-centre sample investigated in this study is double the size of the Canadian sample and has sufficient power to detect an association or linkage with similar magnitude to that reported by Quist *et al*. However, we observed no significant transmission of the 452Tyr in the total sample. On the contrary, a significant association between the 452His and ADHD was observed in the Irish sample alone. It is too early to draw any conclusion from these conflicting results and ADHD samples from different ethnic groups are required to solve this discrepancy.

Finally, given the complexity of the serotonin receptors, their linkage to second messenger, their neural distribution and their interaction with other neurotransmitter systems, it is very important to conduct a structural and functional analysis of the HTR_{1B} to assess the contribution of this gene to the development of ADHD.

Acknowledgements

We would like to acknowledge the generous support of the Health Research Board, Dublin, The Wellcome Trust (ZH and AK), and the Hyperactive and Attention Disorder (HAD) Group Ireland. The London group is supported by the MRC. SC and LK are Wellcome Trust Research Fellows. The Cardiff group is also supported by Wellcome and Action Research. Thanks are also due to Jane Holmes and Tracey Heyer who were involved in the first wave of sample collection at Manchester. We would also like to thank the families who participated in the study.

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