Association of serotonin transporter polymorphisms with responsiveness to adrenocorticotropic hormone in infantile spasm

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Background: **Serotonin or 5-hydroxytryptamine (5-HT) is an important neurotransmitter in the central nervous system. The serotonin transporter (5-HTT) is a key regulator of the level of serotonergic neurotransmission. In the present study, the contribution of 5-HTT polymorphisms to the risk of infantile spasm (IS) and the responsiveness to adrenocorticotropic hormone (ACTH) were investigated.**

Methods: **Two functional polymorphisms, the 44-bp insertion-deletion polymorphism in the promoter region *(5-HTTLPR*) and the variable number tandem repeat in the second intron (*5-HTTVNTR*), were genotyped in a Chinese case-control study involving 112 patients with IS and 120 controls.**

Results: **Genotyping yielded valid data in 111 patients and 118 controls for *5-HTTLPR* and 110 patients and 118 controls for *5-HTTVNTR*. The polymorphisms were not found to have an allelic or genotypic association with IS. However, responsiveness to ACTH was higher in patients who were homozygous for L (91%) than in those with S/L (56%) or S/S (60%) (*P*=0.017). Haplotype analysis did not improve the observed association.**

Conclusions: **The results suggest that the *5-HTTLPR* genotype may influence the responsiveness to ACTH. This interpretation deserves further study.**

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Introduction

Infantile spasm (IS), the most common type of early epileptic encephalopathies, is very difficult to control and has poor long-term prognosis. Approximately 200 factors are believed to be related to IS.[1] While several hypotheses have been proposed for the pathogenesis of IS, it remains poorly understood. One such hypothesis is the serotonin or 5-hydroxytryptamine (5-HT)-kynurenine hypothesis, which is based on observations of decreased cerebrospinal fluid levels of 5-hydroxyindoleacetic acid (5-HIAA) and other tryptophan metabolites.[2,3] 5-HIAA is the major metabolite of 5-HT, an important neurotransmitter in the central nervous system (CNS) that plays various roles in the neurodevelopment and plasticity of the brain.

We found that some mothers of IS patients experienced prenatal stress events in a long-term clinical study. Using epidemiological methods, we identified higher levels of maternal prenatal stress in the IS group than in other epilepsy (positive control) or normal (negative control) groups.[4] The distribution of causative factors was not different significantly. We found a significant difference in the number of seizures between pups with prenatal stress exposure and those without (data not published). The aforementioned findings indicate that prenatal stress plays an important role in the onset of IS.

The mechanisms of the impact of maternal prenatal stress on infants involve multiple aspects. 5-HT may be one of the important aspects because the serotonergic system has been widely implicated in stress-related

disorders such as depression and anxiety.

The 5-HT transporter (5-HTT) is a key regulator for the level of serotonergic neurotransmission.[5] The action of 5-HT after its release by presynaptic neurons is terminated primarily by its re-uptake via 5-HTT. The human *5-HTT* is mapped to chromosome 17q11.1-17q12, which spans approximately 35 kb and contains 14 exons. The *5-HTT* locus has two functional polymorphisms: 44-bp insertion-deletion polymorphism in the promoter region (*5-HTTLPR*)[6] and variable number tandem repeat (VNTR) in the second intron (*5-HTTVNTR*).[7] The allelic difference is weak in these polymorphisms but significant in 5-HTT mRNA levels.[8]

Numerous studies have investigated the link between the allelic variations of *5-HTT* (*5-HTTLPR* and *5-HTTVNTR*) and depression- or stress-related phenotypes. Caspi et al[9] found that an increasing frequency of stressful life events and/or the presence of childhood maltreatment are associated with depression and suicidal risks, which are highest in individuals with genotype S/S, moderate for L/S individuals, and lowest for L-homozygotes. In addition, Manna et al[10] found that the *5-HTT* gene may plays a role in the etiology of temporal lobe epilepsy (TLE). However, information on the contribution of these polymorphisms to the risk of IS is scarce.

IS is a seizure refractory to classic anti-epileptic therapy. Adrenocorticotropic hormone (ACTH) has been proven as an effective drug for IS treatment. However, some patients are clinically resistant to ACTH therapy, and the exact molecular mechanism of action of ACTH is not well understood. ACTH, the principal regulator of the hypothalamus-pituitary-adrenal (HPA) axis, stimulates adrenal glucocorticoid (GC) biosynthesis and secretion. A study[11] showed that dexamethasone modulates 5-HTT activity.

The aforementioned studies indicate that *5-HTT* polymorphisms may be involved in the regulation of 5-HT expression and HPA activity. Thus, *5-HTT* may be a candidate gene that influences susceptibility to IS. In the present study, we investigated the effects of the polymorphism in the promoter region as well as the VNTR polymorphism in intron 2 of the 5-HTT gene on IS.

Methods

Participants

A total of 112 ethic Han patients with IS (73 males, 39 females; mean age=6.8 months, SD=3.4) who had been admitted to Beijing Children's Hospital and Chinese PLA General Hospital between 2004 and 2010 were enrolled in the present study. The control group, matched with the patients in terms of sex, age, and ethnicity, consisted of 120 healthy children (80 males, 40 females; mean age=7.5 months, SD=3.9) without neurological disorders. No statistical difference was found between the sexes and ages ($P>0.05$) of the patients and the controls. Diagnosis of IS was based on the criteria proposed by the International League Against Epilepsy.[12] The exclusion criteria included: (1) individuals who had received hormone therapy within 28 days before recruitment; (2) those who were contraindicated for hormone therapy, including a lethal or potentially lethal disease other than IS; (3) the parents or guardians of the patients were not able to provide informed consent. Informed consents were obtained from the parents or guardians. This study was approved by the Ethics Review Committee of Beijing Children's Hospital affiliated to Capital University of Medical Sciences.

Evaluation of treatment and drug responsiveness

Among the 112 patients with IS, 84 received ACTH (Shanghai No.1 Biochemical & Pharmaceutical Co., Ltd., Shanghai, China) treatment (150 U/m^2/d) for 14 days. The remaining 28 patients were treated with other anti-epileptic drugs. All patients underwent a 24-hour examination and/or video-electroencephalography (EEG) before the ACTH therapy to ascertain the presence of hypsarrhythmia or its variants. The assessment criteria for ACTH responsiveness were based on the recommendations by Lux et al[13] and Baram et al.[14] The response was defined as both a complete cessation of clinical spasms (after 14 days of ACTH treatment or a period of 28 days from the time of the last observed IS) and a resolution of the hypsarrhythmic pattern in both asleep and awake EEG. The EEG was evaluated by an investigator who was not informed of the treatment. Patients with persistent IS or hypsarrhythmia were treated with alternative drugs.

Genotyping

Genomic DNAs were prepared from ethylenediaminetetraacetic acid-treated whole blood samples using the standard methods. Polymerase chain reaction (PCR) was used to amplify two polymorphisms in the *5-HTT* gene (*5-HTTLPR* and *5-HTTVNTR*) using the following primers: *5-HTTLPR*, 5'-FAM-CGGGATGCGGGGGAATACTGGT-3' and 5'-GTTTCTTTTGCCGCTCTGAATGCCAGCAC-3'; *5-HTTVNTR*, 5'-FAM-TGGCGAGATTTGACTTTTCTACC-3' and 5'-GTTTCTTCTGAGCTTCATCAAGGGGAAC-3'. The forward primer was 5' labeled with carboxyfluorescein (6-FAM) dye. All PCR products

were then electrophoresed on an ABI 3730 Genetic Analyzer (Applera Corp.). PCR fragments were electrophoresed using GeneScan-500 LIZ (Applera Corp.) as an internal lane size standard. Sequencing was carried out using GeneMapper software version 4.0, as described in the manufacturer's manual.

Statistical analysis

The Hardy-Weinberg equilibrium test was performed. Statistical analysis was made using SNPStats and SPSS (Version 11.5, SPSS Inc., Chicago, IL, USA). Clinical data such as age at onset and response to ACTH treatment were analyzed using the Chi-square test or Fisher's exact test. All tests were two-tailed, and the nominal significance threshold was set at $P<0.05$.

Results

5-HTTLPR

After typing the quality control, valid genotyping data from 111 patients and 118 controls were obtained for further analysis, showing the Hardy-Weinberg equilibrium ($P>0.05$). No significant difference was observed in either the allelic or genotypic frequencies of *5-HTTLPR* between the IS patients and controls (Table 1). However, under the assumption of the recessive model, L/L carriers were more common than L/S or S/S carriers ($P=0.05$), with a relative risk for the L/L genotype of 3.12.

We then determined whether the polymorphism was associated with responsiveness to ACTH therapy. Patients homozygous for the L allele responded to ACTH better than the L/S and S/S carriers, resulting in a significant association ($P=0.017$, OR=7.418) (Table 2, Fig). Among 11 patients with L/L allele, 10 were males and one female with onset age ranging from 3 months to 21 months. Three of the 11 patients were cryptogenic and 8 symptomatic.

5-HTTVNTR

Genotyping yielded valid data in 110 patients and 118 controls. Association analysis showed no significant differences in either the allelic or genotypic frequencies of *5-HTTVNTR* between the patients and controls ($P>0.05$). Responsiveness to ACTH was not associated with this marker ($P>0.05$).

Haplotype analysis

Haplotype analysis did not show any association of haplotypes with IS or with responsiveness to ACTH.

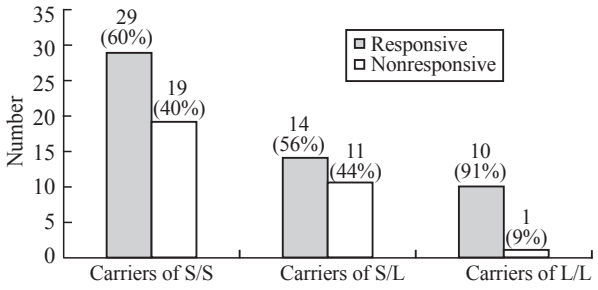

Fig. Comparison of adrenocorticotropic hormone (ACTH) responses between different phenotypes of 44-bp insertion-deletion polymorphism in the promoter region (*5-HTTLPR)* in patients who received ACTH therapy. The response rate of L/L carriers is significantly higher than that of L/S or in S/S carriers.

Table 1. Comparison of genotype and allele frequencies of *5-HTTLPR* in patients with infantile spasm and healthy controls

5-HTTLPR	Patients	Controls	95% CI	Individual *P*-value	Overall *P*-value
Genotype	*n*=111	*n*=118			
S/S	63 (56.8%)	72 (61.0%)	1	0.95	0.11
S/L	35 (31.5%)	41 (34.8%)	0.764 (0.389-1.500)	0.25	
L/L	13 (11.7%)	5 (4.2%)	3.120 (0.851-11.442)	0.05	
Allele	*n*=222	*n*=236			
S	161 (72.5%)	185 (78.4%)	1	0.43	0.43
L	61 (27.5%)	51 (21.6%)	1.227 (0.738-2.041)		

CI: confidence interval; *5-HTTLPR*: 44-bp insertion-deletion polymorphism in the promoter region.

Table 2. Adrenocorticotropic hormone responsiveness in patients carrying different genotype and allele of *5-HTTLPR*

5-HTTLPR	Response	No response	95% CI	Individual *P*-value	Overall *P*-value
Genotype	*n*=53	*n*=31			
S/S	29 (54.7%)	19 (61.3%)	1	0.59	0.049
S/L	14 (26.4%)	11 (35.5%)	0.744 (0.271-2.040)	0.30	
L/L	10 (18.9%)	1 (3.2%)	7.418 (0.861-63.921)	0.017	
Allele	*n*=106	*n*=62			
S	72 (67.9%)	49 (79.0%)	1	0.12	0.11
L	34 (32.1%)	13 (21.0%)	1.796 (0.858-3.760)		

CI: confidence interval; *5-HTTLPR*: 44-bp insertion-deletion polymorphism in the promoter region.

Discussion

Increasing evidence links *5-HTT* polymorphisms to complex disorders,[15] including epilepsy. However, information about whether these polymorphisms contribute to the risk of IS is scant. In the present study, we analyzed two polymorphisms in IS patients and controls. One marker, HTTLPR, was found to be associated with the responsiveness to ACTH. About 91% (10/11) of L/L carriers responded to ACTH, compared with 56% (14/25) of L/S carriers and 60% (29/48) of S/S carriers ($P<0.05$). These findings suggest that the *5-HTT* variant may influence the responsiveness to ACTH, rather than the risk of IS. Otherwise, patients with the L/L genotype may also respond well to vigabatrin or some other treatment; this hypothesis requires further study.

Approximately 200 factors are believed to be related to IS. However, not all patients develop IS. An example is brain hypoxia-ischemia. The diverse etiologies resulting in IS must share at least one common feature. Given this diversity, we did not divide the IS patients into subgroups according to etiology. However, one factor responsible for infantile spasm may well affect response to ACTH (e.g. patients with tuberous sclerosis, known genetic mutations causing early onset epileptic encephalopathy, etc.). Thus, lack of etiology data for symptomatic patients and relationship to ACTH response was a limitation of this study.

5-HTT plays a pivotal role in the regulation of 5-HT. *5-HTT* genetic variations affect the basal cerebral metabolic activity in limbic structures in a normal population,[16] and are associated with various disorders.[9,17] Manna et al[10] reported a negative association of allele 10 of the 5-HTT marker, *5-HTTVNTR*, with TLE patients. This finding suggests the genetic importance of the *5-HTT* gene in TLE development. More recently, Hecimovic et al[18] found that the combination of *5-HTT* genotypes linked with higher *5-HTT* gene expression (*5-HTTLPR* L/L and VNTR-2 12/12) was associated with a worse response to optimal drug therapy. Our results were inconsistent with the data obtained by Hecimovic et al,[18] which may have resulted from the distinctive efficacy of ACTH in IS.

The mechanisms underlying the effects of *5-HTTLPR* on the responsiveness to ACTH may involve multiple aspects. First, the L/L homozygous genotype produces differential levels of 5-HT because of the altered 5-HTT expression and activity. 5-HT is one of the neurotransmitters influencing the cortical and subcortical excitatory/inhibitory balance. It participates in many physiological and pathological processes of the brain. The alteration of expression levels may partly contribute to the different responses to ACTH. Second, ACTH stimulates the adrenal GC biosynthesis and secretion via the specific cell-surface melanocortin 2 receptor (MC2R). Glatz et al[11] found that synthetic GC dexamethasone administration results in an allele-dependent increase in *5-HTT* gene activity using the luciferase reporter assay. In addition, removing the tandemly repeated human promoter element led to a loss of luciferase activity in response to glucocorticosteroid hormone administration. Third, only two polymorphisms of *5-HTT* were investigated in the present study, and they may not fully cover the *5-HTT* gene or other genes. Thus, additional genetic markers of the 5-HT receptors may be required to fully assess whether *5-HTTLPR* influences ACTH efficacy.

Previously, we reported that both polymorphisms of the *MC2R* promoter[19] and haplotypes of the G protein-regulated inducer of neurite outgrowth 1[20] are important factors influencing the efficacy of ACTH therapy. Multiple factors contribute to the susceptibility to IS development and treatment responsiveness. Hence, different factors may influence ACTH efficacy via the "final common pathway". Further studies should focus on finding a common pathway, which may help to elucidate the pathogenesis of IS.

We are aware of the limitations and subtle biases of any association study that requires replication using larger sample sets. Children with early onset IS (particularly if they are secondary to epileptic encephalopathies, such as CDKL5 mutations, etc.) and delayed treatment have been reported to be much less responsive to any treatment, including ACTH. A recent study[21] suggests that both prompt diagnosis and prompt treatment of IS may help to prevent subsequent developmental delay. We did not have detailed information about several patients, which poses another limitation to this study. Further genetic and molecular studies are required to fully understand the potential etiology of cryptogenic patients.

In summary, the specific phenotypes (L/L) of *5-HTTLPR* may influence responsiveness to ACTH and other treatments. These findings provide clinicians with an early predictive marker for responsiveness to ACTH and improve our understanding of serotonergic mechanisms in epileptogenesis. Further study is necessary to confirm our findings.

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Competing interest: None declared.

Contributors: Shi XY and Zou LP did laboratory work (PCR, sequencing) and drafted the manuscript. Yang G, Ding YX, Sun YH and Jia FY collected clinical data. He B made data anylasis. All authors approved the final version of the article for publication.

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