0021-972X/06/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 91(6):2432–2436

doi: 10.1210/jc.2005-2657

BRIEF REPORT

Allelic Variants of the γ-Aminobutyric Acid-A Receptor α1-Subunit Gene (*GABRA1*) Are Not Associated with Idiopathic Gonadotropin-Dependent Precocious Puberty in Girls with and without Electroencephalographic Abnormalities

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Context: γ-Aminobutyric acid (GABA) is a dominant inhibitory neurotransmitter involved in the modulation of brain electric activity and puberty onset in primates. GABA inhibitory effects on GnRH neurons are mainly mediated by GABA-A receptor α1-subunit.

Objective: The objective of this study was to investigate functional mutations or polymorphisms of the GABA-A receptor α1-subunit gene (*GABRA1*) in girls with idiopathic gonadotropin-dependent precocious puberty (GDPP) with and without electroencephalographic (EEG) abnormalities.

Design: The entire coding region of *GABRA1* was sequenced in all patients. Two known *GABRA1* polymorphisms were investigated by GeneScan software analysis or enzymatic restriction. Seventy-three normal women were used as controls for genetic study. EEG tracings were recorded in 23 girls with GDPP and 17 girls with adequate pubertal development.

Setting: The study was performed at a university hospital.

Patients: Thirty-one girls from 28 unrelated families with idiopathic GDPP were studied.

Results: Automatic sequencing revealed no functional mutations in girls with GDPP. Seven different *GABRA1* polymorphisms, including two exonic (156T>C and 1323G>A) and five intronic [IVS2–712(GT)n, IVS3+12A>T, IVS8+45T>G, IVS9+76A>G, and IVS10+15G>A], were found in GDPP girls and controls. Abnormal EEG tracings were found in 26% of 23 girls with GDPP, two of them with epilepsy. The genotype and allele frequencies of the *GABRA1* polymorphisms were not statistically different between unrelated GDPP girls and controls or between GDPP girls with or without EEG abnormalities.

Conclusions: *GABRA1* functional mutations or polymorphisms are not associated with the intrinsic mechanism of GDPP in girls with and without EEG abnormalities. **(*J Clin Endocrinol Metab* 91: 2432–2436, 2006)**

PUBERTY IS INITIATED by the reemergence of the pulsatile secretion of GnRH after a quiescent period during childhood controlled by several inhibitory factors (1, 2). γ-Aminobutyric acid (GABA) is a dominant inhibitory neurotransmitter that has been involved in the intrinsic mechanism of the onset of human puberty (1–4). Three distinct receptors (A, B, and C) mediate GABA actions (5). The GABA-A receptors are ligand-gated ion channels that consist of at least 18 different subunits (α1-α6, β1-β4, γ1-γ4, δ, π, and ρ1-ρ2) organized in a heteropentameric form (2, 5, 6). The GABA-A receptor α1-subunit is related with the inhibitory activity of GABA on GnRH release, and its high expression during postnatal development in rats indicates brain maturation, *i.e.* the onset of GABAergic synaptic inhibition (7, 8).

GABA is also involved in the modulation of brain electric activity (9). Interestingly, electroencephalographic (EEG) abnormalities have been described in patients with organic and idiopathic gonadotropin-dependent precocious puberty (GDPP) (10, 11). To date, no molecular analysis of the GABA receptor has been performed in human central pubertal disorders. In this study we hypothesized that functional mutations or polymorphisms in the GABA-A receptor α1-subunit gene (*GABRA1*) might confer susceptibility to GDPP.

Subjects and Methods

The protocol was approved by the ethical committee of Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo. Twenty-eight unrelated girls with idiopathic GDPP were selected for

First Published Online March 28, 2006

Abbreviations: EEG, Electroencephalograph; GABA, γ-aminobutyric acid; *GABRA1*, GABA-A receptor α1-subunit gene; GDPP, gonadotropin-dependent precocious puberty; IFMA, immunofluorometric assay.

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this study, and written informed consent was obtained from their parents. Clinical and hormonal data of these patients are shown in Table 1. Three girls (patients 26–28) had a familial form of GDPP, and their affected relatives from the same generation were also studied (patients 29–31). The onset of pubertal signs ranged from 0.3–7 yr (mean, 4.7 ± 2.1 yr). All patients had breast development ranging from Tanner stages 2–4 and pubic hair from Tanner stages 1–5 (12). High growth velocity and advanced bone age (Greulich and Pyle method) were observed in all patients. Basal and/or stimulated LH levels after classical GnRH test or 2 h after depot leuprolide administration were at pubertal levels according to the previously established cutoff values for immunofluorometric assay (IFMA) or RIA methods (13, 14). Basal LH levels were at pubertal levels (>0.6 U/liter for IFMA) in 20 of 30 patients with GDPP (Table 1). Twenty-two patients showed an LH peak at pubertal levels (>6.9 U/liter for IFMA or >15 U/liter for RIA) after a classical GnRH stimulation test. Two patients (patients 16 and 25) who showed a prepubertal response to the classical GnRH test had a pubertal LH response (>10 U/liter) 2 h after depot leuprolide administration (14). In addition, all girls achieved a satisfactory therapeutic response to depot GnRH analogs.

All patients had normal central nervous system magnetic resonance imaging, indicating the idiopathic form of GDPP. Two girls (patients 14 and 23) had had a previous diagnosis of idiopathic generalized epilepsy and were being treated with valproic acid.

DNA analysis

GABRA1 is located at chromosome 5q34–35 and contains 11 exons (15). Exons 3–11 codify the protein with 456 amino acids. The *GABRA1* genomic structure was based on the National Center of Biotechnology Information database (www.ncbi.nlm.nih.gov; GenBank accession no. NM_000806; Gene ID 2554).

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures. Specific intronic oligonucleotides for *GABRA1* amplification were obtained using Primer3InPut software (http://fokker.wi.mit.edu/primer3; Table 2). *GABRA1* amplification PCR and sequencing protocols are available upon request.

Two *GABRA1* polymorphisms, IVS2–712(GT)n and 1323G>A, were also studied using GeneScan software (Applied Biosystems, Foster City, CA) and a PCR-based assay in which amplified DNA was digested with the restriction endonuclease *Tai*I (MBI Fermentas, Hanover, MD), respectively.

Seventy-three normal fertile women (18–40 yr) with pubertal development at appropriate chronological age (mean age of menarche, 12.5 ± 1.2 yr) were used as controls for genetic studies.

Electroencephalographic study

Twenty-three of the 31 girls with GDPP were submitted to EEG. Additionally, 17 girls with pubertal stage congruent with chronological age (mean age, 10.8 ± 3.2 yr; ranging from 6–15 yr), without history of epileptic seizures, underwent EEG due to migraine investigation, and their EEG tracings were analyzed retrospectively.

EEG was performed according to international standards with 18-channel analogical (Neurofax, Nihon Kohden) or 19-channel digital (Neuromap, Neurotec) equipment. The EEG tracings were recorded after sleep deprivation. Electrode system placement was in accordance with the International 10–20 system. The sensitivity was set at 50 μV/

TABLE 1. Clinical data and basal and GnRH-stimulated gonadotropin levels of 31 girls with idiopathic GDPP

Patient no.	CA at pubertal onset (yr)	CA at first visit (yr)	BA (yr)	Pubertal stage (Tanner)	LH (U/liter)		FSH (U/liter)		Estradiol (pg/ml)
					Basal	Peak[a]	Basal	Peak[a]	
1	6.0	7.33	9	B3PH3	<0.6	8.1	1.3	6.7	<13
2	4.0	5.25	6.83	B3PH1	<0.6	16.9	2.1	20.4	<13
3	6.0	8.66	12	B4PH4	1.2		3.7		19.8
4	6.25	6.66	10	B2PH1	1.5	6.9	2.8	9.9	21.1
5	6.5	8.08	12	B3PH3	0.7	7.5	3	5.2	45
6	1.25	2.66	6.83	B4PH4	2.6	30	4.5	16	<10[b]
7	0.66	1.66	2	B4PH3	1.1		4.3		60.4
8	3.17	3.42	6.83	B4PH1	5		9.8		<13
9	6.42	7.83	11	B3PH4	1	13	2.8	18	30[b]
10	2.33	7.25	11	B3PH2	0.9		1.8		<13
11	6.0	6.42	8.83	B2PH2	<0.6	9.5	4.7	24	<10[b]
12	2.33	4.75	8.83	B4PH1	0.8	18.8	3.3	9.9	19.1
13	3.17	5.17	12	B4PH3	4.9	23.7	5.9	12.9	14[b]
14	7.0	7.83	11	B3PH1	2.8	15	7.1	10.3	59.8
15	5.83	9.66	12	B4PH3	0.7		1.5	13.5	<13
16	3.17	6.5	8.83	B4PH3	<0.6	4.2	3.7	10.5	<13
17	6.66	7.33	10	B3PH2	1.4	20	1.8	7.5	<13
18	6.83	7.83	8.83	B3PH3	2.8	41	4.5	16	<10[b]
19	4.0	6.83	9.5	B4PH3	6.1		4.7		62
20	2.33	7.42	11	B2PH4	2.2	9.8	6	11	21
21	0.3	8.58	11	B4PH2	<0.6	8.5	1.5	16.9	22
22	7.0	8.75	11.5	B3PH3	1.2	19	1.8	5.4	13.1
23	6.0	8	11	B3PH3	1.0	8.8	1.9	9.5	17.4
24	6.0	4.83	8	B3PH1	3.8[b]	28.8[b]	16.3[b]	41.4[b]	23[b]
25	2.0	8.25	12	B3PH2	<0.6	5.3	2.7	25.8	13.4
26[(F1)c]	6.5	10.25	11	B4PH5	4.2		2.6		98.8
27[(F2)c]	5.0	8	11	B4PH1	1.2	19	1.8	5.4	13.1
28[(F3)c]	5.5	8.08	11	B3PH4	<0.6	8.5	2.6	13	22
29[(F1)]	7.0	9.83	11.5	B4PH4	1.7	34.1	3.9	11.3	20.2
30[(F2)]	5.83	6.66	11	B4PH2	1.5	31.5	6.2	12.8	53.3
31[(F2)]	4.0	6.42	7	B3PH1	<0.6	11.4	1.5	12.7	<13
Mean ± SD	4.7 ± 2.1	7 ± 2	9.8 ± 2.2		1.7 ± 1.5	16.1 ± 10	3.5 ± 2	12.7 ± 5.5	27 ± 22
(range)	(0.3–7)	(1.66–10.2)	(2–12)		(<0.6 to 6.1)	(4.2–41)	(1.3–9.8)	(5–25)	(<13 to 98)

F1, F2, and F3 are familial cases. B, Breast development; BA, bone age; CA, chronological age; PH, pubic hair.
[a] Classical GnRH (100 μg, iv) stimulation test: blood collection at 0, 15, 30, 45, and 60 min.
[b] Levels were measured by RIA and were not included in mean ± SD calculation.
[c] Index cases.

TABLE 2. Intronic oligonucleotides used in this study

Exon	Annealing temperature (C)	Fragment size (bp)	Primer	Nucleotide sequences (5′-3′)
E3	55	297	F	CAGTCAGCCCTGGTGGTTAT
			R	TAGCTGGAAATTATTGCAGTTAAG
E4	55	498	F	TGGAAAGAGGTTTTAGTAGAAATGTAT
			R	GCAGTCATTGTGCTGGAAGA
E5	51	379	F	GACACTCACTCGCCCAATTT
			R	GGGGTATTAACCAATTCAAA
E6	55	372	F	GCATTGCAAAATACAGCACA
			R	CAAATATTCCACCATGGCTCA
E7	55	290	F	CCCTGCAATTATGCCTTCTT
			R	CCTTATCTACGCGTTTTTCTCAC
E8	53	501	F	AATTGAAGTGGTAAAATATATGGATCA
			R	GGGGAATAAGGATTTAACCCAG
E9	55	231	F	CAAATTGCTCATCTTTCTTGTG
			R	TGAGAGTGGCAATTCCTTGA
E10	55	544	F	TGCCATTCCATGAATCACAG
			R	TCATGGCACTTAATTGTTTACG
E11	55	660	F	TTCTTGATGGCAAAAGGCTA
			R	CAGGGGTCTCTTGTCTTAAATGA
I 2	55	247	F	TCCAGCTTCCATCTGTTTGA
			R	CCGGAGTCGTGCTTTTATTC

E, Exon; F, forward; I, intron; R, reverse.

mm, with low-frequency 0.5-Hz filters and a high-frequency 70-Hz filter. EEG tracing, recorded in wakefulness, drowsiness, and sleep, lasted 30 min, with 3 min of hyperventilation and intermittent photic stimulation with frequencies of 3, 6, 9, 12, 15, 18, and 24 flashes/sec. The identified EGG abnormalities were confirmed in a second recording. All EEG tracing analyses were performed by the same neurologist (L.M.F.F.G.).

Statistical analysis

Hardy-Weinberg proportions and linkage disequilibrium values were estimated and tested through appropriate software created by Dr. Paulo A. Otto (University of Sao Paulo, Sao Paulo, Brazil).

Comparisons between unrelated patients with GDPP and controls were performed using contingency tables by Fisher's exact test or χ^2 analysis for each polymorphism by Instat software (GraphPad, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant. Comparison of allele and genotype distributions between unrelated GDPP patients with and without EEG abnormalities was performed using contingency tables by Fisher's exact test or χ^2 analysis, followed by Bonferroni-adjustment, with P values adjusted according to the number of comparisons; $P < 0.004$ was considered significant (P corrected).

Results

DNA analysis

Automatic sequencing did not reveal potential functional mutations of the *GABRA1* in girls with GDPP. Seven different polymorphisms were identified: two silent polymorphisms, 156T>C and 1323G>A, located at exons 4 and 11, respectively, and five intronic polymorphisms: IVS2–712(GT)n, IVS3+12A>T, IVS8+45T>G, IVS9+76A>G, and IVS10+15G>A in both girls with GDPP and controls. Genotype distributions among patients and controls were in Hardy-Weinberg equilibrium. No linkage disequilibrium was found among all *GABRA1* polymorphisms.

The allele and genotype distributions of the two exonic polymorphisms of the *GABRA1* (156T>C and 1323G>A) did not differ between GDPP patients and 50 controls ($P = 0.85$ and 0.45 for allele frequencies and $P = 0.92$ and 0.44 for genotype distributions, respectively). We identified 12 different alleles and 28 genotypes for the IVS2–712(GT)n polymorphism in the patients and 73 controls. The distribution of allele frequencies for this polymorphism also did not differ between girls with idiopathic GDPP and the 73 controls ($P > 0.05$ for all comparisons). However, we observed that in the IVS2–712(GT) polymorphism, the alleles with 12 and 17 repeats were more prevalent in both groups, without a statistically significant difference ($P = 0.15$ and $P = 0.82$, respectively).

Electroencephalographic study

Six of the 23 patients (26%) had EEG abnormalities that were confirmed by a second tracing. Two of them (patients 14 and 23) had epilepsy, and their EEGs disclosed generalized spike-wave complexes in one and focal discharge in another. The remaining four patients did not have epilepsy (two of them sisters, patients 27 and 31; Table 1). The EEG abnormalities in these asymptomatic girls consisted of focal discharges (two of them in posterior regions) and diffuse slowing. The EEG tracings of the 17 girls with adequate pubertal stage showed only one with abnormal paroxysmal activity.

No statistically significant difference was found in the genotype and allele frequencies of polymorphisms of the *GABRA1* between girls with GDPP with and without EEG abnormalities, with $P < 0.004$ (P corrected).

Discussion

The GABAergic inhibition of hypothalamic GnRH neurons mediated through GABA-A receptor α1-subunit appears to be critical to maintain the quiescent normal prepubertal period in females (1–3). Abnormalities of *GABRA1*, such as loss of function mutations or polymorphisms, represent an exciting hypothesis to explain the precocious reemergence of pulsatile pubertal GnRH release in females with idiopathic GDPP. In the present study no functional

mutation of the *GABRA1* was found in 31 girls with sporadic or familial idiopathic GDPP. Seven different *GABRA1* polymorphisms were identified in these patients. Genotype and allele frequencies of the *GABRA1* polymorphisms were not statistically different between unrelated GDPP girls and controls.

To date, only one functional mutation (Ala322Asp) in the *GABRA1* has been detected in a large French-Canadian family with an autosomal dominant form of juvenile myoclonic epilepsy (16). The functional analysis of this mutation showed reduced amplitude of GABA-activated currents *in vitro*, suggesting that seizures may result from the loss of GABA-A receptor function (16). It is noteworthy that EEG abnormalities and epilepsy have been previously associated with idiopathic and organic GDPP (10, 11). Hypothalamic hamartomas represent the most frequent known organic causes of GDPP, often associated with gelastic seizures. Epileptogenesis mechanisms implicated in this subcortical lesion remain unknown (17). However, it was recently demonstrated that hypothalamic hamartoma tissues contained predominantly GABAergic inhibitory neurons that exhibited intrinsic pacemaker-like behavior (17).

In the 1970s, Liu *et al.* (10) verified the prevalence of electroencephalographic abnormalities in 42 children (39 girls) with idiopathic precocious puberty (10). At that time, the idiopathic form was established based on the absence of neurological symptoms. A significantly high proportion (81%) of electroencephalographic tracings was clearly abnormal (slow-wave and/or paroxysmal activity) in these children (10). More recently, Theodore *et al.* (11) studied the EEGs of 16 children with precocious puberty, eight of them considered to have the idiopathic form (11). The EEG was abnormal in seven patients, including four with the idiopathic form of GDPP. These findings suggested that the abnormal EEG in these patients could reflect occult diencephalic disease (11).

In this study EEG abnormalities were demonstrated in 26% of girls with idiopathic GDPP. Two of them had generalized epilepsy controlled by antiepileptic drugs (valproic acid). Valproic acid can interfere with pubertal maturation and induces endocrine abnormalities (18). It has been proposed that its antireproductive effects in rodents are related to enhancing GABAergic inhibition of the GnRH neuronal population within the medial preoptic area, but they are still poorly understood (18). The other four girls with EEG abnormalities had no apparent neurological disorder. Interestingly, two neurological asymptomatic girls with EEG abnormalities were sisters, suggesting that the phenotype composed by abnormal EEG and idiopathic GDPP could be modulated by genetic factors. However, we did not demonstrate a statistically significant difference in distribution of the *GABRA1* polymorphisms in GDPP girls with or without EEG abnormalities. Additionally, a normative database for EEG indicates age-related maturational changes and sex differences in the EEG patterns (19). Cavazzuti *et al.* (20) described EEG abnormalities in 3.54% of 3726 children who were neurologically normal and had no history of epileptic seizures. In our study we demonstrated EEG abnormalities in 5.8% of the girls with adequate pubertal stage.

Although we were not able to correlate molecular defects in the *GABRA1* with EEG abnormalities in girls with GDPP, other GABA receptor subunits should be investigated to definitely exclude this association, because these EEG abnormalities are presumed to be caused by an age-dependent cortical hyperexcitability, in which GABA has an important role (9). Neurological and clinical follow-up of girls with GDPP associated with EEG abnormalities is worthwhile, because their importance and consequences remain to be elucidated.

In conclusion, *GABRA1* functional mutations or polymorphisms were not associated with the intrinsic mechanism of GDPP in girls with and without EEG abnormalities.

Acknowledgments

We thank Dr. Paulo A. Otto (Department of Genetics and Evolutionary Biology, Sao Paulo University), the staff of Laboratório de Hormônios e Genética Molecular/LIM 42, and Ms. Sonia Strong for the English review.

Received December 9, 2005. Accepted March 20, 2006.

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This work was supported in part by grants from Fundação Faculdade de Medicina da Universidade de São Paulo (to V.N.B.).

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