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Polymorphism in genes involved in adrenergic signaling associated with Alzheimer's

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Abstract

To investigate the potential involvement of adrenergic signaling in Alzheimer's disease (AD) pathogenesis, we performed genetic and functional studies of genes initiating the cascade. We chose two functional single-nucleotide polymorphisms (SNPs) in the β1-adrenergic receptor (*ADRB1*) and the G protein β3 subunit (*GNB3*) genes, respectively, and analyzed their allelic frequencies in a case-control sample of AD. We found that the *GNB3* T allele produces a significant risk for AD in individuals homozygous for the *ADRB1* C allele, suggesting that the combined effect of both polymorphisms influences AD susceptibility. Interestingly, the co-expression of *GNB3* T and *ADRB1* C alleles, compared with *GNB3* C and *ADRB1* G, produced increased cAMP levels and MAPK activation following adrenergic stimulation of transfected human cell lines. Furthermore, the co-expression of these alleles also produced increases in APP expression. These data strongly indicate that the combination of *GNB3* and *ADRB1* polymorphisms produces AD susceptibility by changing the cell responsiveness to adrenergic stimulation, pointing to the modulation of brain adrenergic receptors as a potential target for novel AD therapeutic strategies.

Keywords: G β3 subunit; β1 adrenergic receptor; Polymorphism; Neuronal adrenergic signaling; cAMP; APP expression; MAPKs; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive decline in memory and other intellectual functions, which in most cases presents as a complex trait resulting from as yet unknown combination of genes and environmental factors. The ε4 allele of apolipoprotein E (*APOE* ε4) is the main genetic risk factor for AD identified to date [6], although it is widely accepted that other genes should be involved in AD susceptibility [27]. There is also strong evidence that environmental factors, remarkably severe head injury, may modify the *APOE* related risk [11,28].

The beta1-adrenergic receptor (β1AR), member of the G-protein-coupled receptor family [17], is the most abundant β-adrenergic receptor subtype in the mammalian brain, and exhibits a predominantly neuronal expression pattern [5]. β1AR activation promotes Gs-mediated stimulation of adenylyl cyclase leading to an increase in intracellular concentrations of cyclic adenosine 3′,5′-monophosphate (cAMP). Recently, a polymorphism in a G-protein coupling domain of the human β1AR has been reported [20,23]; this single nucleotide polymorphism consists in a change of G to C at nucleotide position 1165, resulting in a substitution of Gly to Arg at position 389 of the protein sequence. This residue is located at a highly conserved region critical for G-protein coupling, and the Arg-389 isoform of the receptor is associated with enhanced β1AR-G-protein interaction resulting in an increased activation of the adenylyl cyclase effectors [23]. Interestingly, the G protein beta subunits have been reported to play an important role in the coupling of Gs to the β1AR [24]. Siffert and his collaborators [33] have identified a C/T polymorphism at nucleotide position 825 of the gene encoding the beta3 subunit of heterotrimeric

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G proteins, the G-protein β3 gene (*GNB3*). This polymorphism does not affect the amino acid sequence of Gβ3, but the 825T allele results in an alternative splicing variant (*GNB3*-s) which may enhance signal transduction. Since adrenergic activation takes place during head injury [29], which is the major non-genetic risk-factor identified to date for AD [26], we have explored a potential relationship between polymorphisms in the genes of these key components of adrenergic signaling, and the occurrence of AD. We have found that a given combination of these polymorphisms results in increased response to β-agonists, enhanced amyloid precursor protein (APP) expression in response to adrenergic stimulation, and susceptibility to Alzheimer's disease.

2. Materials and methods

2.1. Subjects

We have studied a case-control sample of 348 individuals, integrated by 175 late-onset sporadic AD cases (age at onset 72.4±5.2 years; mean±S.D.) from the central area of Spain, recruited through Hospital Neurology Services and 173 healthy controls (age at examination 73.3 ± 5.3 years). Cases were clinically diagnosed as probable AD following the NINCDS-ADRA [25] or DSMIV [35] criteria for Alzheimer's dementia and controls were subjected to a Mini Mental test. This sample has been previously genotyped for *APOE* and other polymorphic genes [3,4,14].

2.2. Genotyping

We selected two functional single nucleotide polymorphisms (SNPs), located in the coding region of the *ADRB1* and *GNB3* genes, respectively. The *ADRB1* polymorphism (dbSNP database::rs1801253) consisted in a C/G change at the first nucleotide of codon 389, coding for the Arg389 and Gly389 protein isoforms, respectively. The *GNB3* polymorphism (dbSNP::rs5443, named C825T in [33]) is a C/T change in exon 10; the T allele generates a splice variant (*GNB3*-s), in which the nucleotides 498–620 of exon 9 are deleted [33]. DNA was extracted from whole blood and genotyping was performed by RFLP using methods described [20,23,33] with minor modifications. Briefly, a *ADRB1* fragment was amplified with primers 5′-CGC TCT GCT GGC TGC CCT TCT TCC (forward) and 5′-TGG GCT TCG AGT TCA CCT GCT ATC (reverse), and digested with *Bst*NI; a *GNB3* fragment was amplified using the primers 5′-TGA CCC ACT TGC CAC CCG TGC and 5′-CTG GGG AGG GTC CTT CCA GCT, and digested with *Bsa*JI.

2.3. Statistical analysis

In the crude analyses, genotype and allele distributions were compared with a χ^2 test, and the strength of the associations was estimated with the odds ratios (OR) with 95% confidence intervals (CI). For the adjusted analyses, we generated a logistic regression model, that included as the independent variables the age at onset, gender, *APOE* (categorized into three groups corresponding to 0, 1 or 2 apoE4 alleles), *ADRB1* (grouped into two categories: CC and (CG + GG) and *GNB3* (0, 1 or 2 T alleles); the model also included interaction terms that were the product of the categories of *GNB3* by *ADRB1*; the strength of association between the *GNB3*/*ADRB1* genotype and AD was summarized with OR and their 95% CI. Analyses were performed with the SAS 8.2 software.

2.4. β1AR and Gβ3 cDNA construction and transfection

The cDNAs coding for *β1AR* (*ADRB1* G allele) and *Gβ3* (*GNB3* C allele) were cloned into *pREP4* (InvitroGen), and site directed mutagenesis was used to obtain the corresponding counterparts (*ADRB1* G and *GNB3* C, respectively). Replacement of the *ADRB1* G (Gly389) by C (Arg389) was performed with the Quickchange site-directed mutagenesis kit (Stratagene) with the primers 5′-CAA GGC CTT CCA GCG ACT GCT CTG CTG-3′ and 5′-CAG CAG AGC AGT CGC TGG AAG GCC TTG-3′. The *GNB3* T allele (splice variant) was derived from the *GNB3* C construct by using a two-step sequential PCR; the first step consisted of the amplification of two partially overlapping fragments, obtained with the primers 5′-GCT GGA AGC TTG CCA TGG GGG AGA TGG AGC A (external upper) and: 5′-CCC AGA GCT TGG CAC ACG TGG TGT CCC CCC GTG C (internal lower) for the 5′ fragment and 5′-GTC GGC TCG AGG TTC ACT GCC TTC CAC TTC C (internal upper) and the 5′-CGG GGA CAC CAC GTG TGC CAA GCT CTG GGA TGT GCG (external lower) for the 3′ fragment; the second round PCR was performed with the external upper and external lower primers. The presence of the expected allelic forms and the absence of additional mutations were confirmed by sequencing all the constructs. Transfection into human embryonic kidney cells (HEK-293) was made with the Lipofectamine plus method (Life Technologies). SK-N-MC were transfected with the Scort II method (Sigma). Stably transfected SK-N-MC cells were selected and maintained with 200 μg/ml hygromycin B (Calbiochem) and 200 μg/ml geneticin (Gibco).

2.5. Adenylyl cyclase assays

HEK-293 and SK-N-MC cells were transfected with the desired combinations of the *ADRB1* and the *GNB3* alleles. After 24 h, cells were split into a 6-well/plate (6×10^5 cells/well), cultured for 12 h and treated with 1 μM of the β-antagonist betaxolol for 2 min, to block basal receptor activity. After extensive washing with phosphate-buffered saline, cells were incubated with either 10 μM of the β-agonist isoproterenol, 10 μM forskolin or vehicle for 10 min. Lysates were obtained and assessed in triplicate for cAMP levels as described [20,23,33]. Data were corrected

according to the total protein amount in each sample, normalized for forskolin activation, and referred to the value obtained in the cells expressing the control isoforms (*ADRB1* G and *GNB3* C alleles). Transfected cells were examined for the expression levels of Gβ3 by Western blot analysis (see below), and of β1AR by radioligand binding experiments with (^{3}H)-dyhydroalprenolol. Similar levels of both proteins were detected for all the transfectants. Radioligand binding experiments were also used to rule out differences in agonist affinity between the β1AR Arg and Gly-389 isoforms (not shown).

2.6. Western blot

Expression levels of APP, Gβ subunits and MAPK were detected by western-blot following standard methods. Briefly, cell extracts were loaded on 10–12% SDS–polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Bio-Rad). Non-specific reactivity was blocked by incubation with BSA 3%/Tween-20 0.2%. Membranes were further processed by incubating with primary antibodies specific for Gβ subunits (anti-human-Gβ3 or anti-Gβcommon polyclonal antibodies, Santa Cruz Biotechnology), MAPK (anti-human-ERK1/2 polyclonal antibody, Cell Signaling Technology), or APP (22C11 mouse monoclonal antibody, Chemicon International) for 2 h at room temperature or overnight at 4 °C, followed by incubation with a secondary antibody (anti-rabbit/goat horseradish peroxidase conjugated, Nordic). Finally, bands were visualized with the enhanced chemiluminiscence Western-blotting analysis system (ECL, Amersham). For internal control, the levels of alfa-tubulin were examined on the same blot using anti-alfa-tubulin (Sigma) and anti-mouse/horse horseradish peroxidase conjugated (Vector) as the secondary antibody. Band intensity was quantified by laser densitometry analysis (BioRad G710).

2.7. MAPK activity

HEK-293 and SK-N-MC cells were transfected with the desired combinations of the *ADRB1* and the *GNB3* alleles. After 36 h of transfection, cells were starved for 2 h, treated with or without 10 μM of isoproterenol for 5 min at 37 °C and resuspended in 0.4 ml of solubilization buffer (200 mM MES, pH 6.2, 1% (v/v) Triton X-100, 0.1 mM $MgCl_2$, 0.3 mM NaCl, 0.1 mM EGTA, 0.5% deoxycholate, 1% *N*-dodecyl-β-D-maltoside and a cocktail of protease inhibitors). After incubation for 2 h at 4 °C, the lysates were clarified by centrifugation at 12,000 × *g* for 15 min. MAPK activation was detected by Western blot analysis using an anti-phospho-p42/p44 MAP kinase (Thr 202/Tyr 204) polyclonal antibody (New England Biolabs). Results were normalized by re-probing the same blots with anti-ERK antibodies as detailed above.

3. Results

The genetic association of the *ADRB1* and *GNB3* polymorphisms with AD was explored in a case-control sample; the sample was an extension of that analyzed previously for *APOE* polymorphisms [3,4] and showed the expected association between ApoE4 and disease (odds ratio for ApoE4 allele 8.7, $P < 0.0001$) as reported previously. The genotype distribution for *ADRB1* and *GNB3* polymorphisms is shown in Table 1. Allele distribution into genotypes was close to that expected under Hardy–Weinberg equilibrium (χ^2 test, $P > 0.5$). As observed in the table, we detected a marginally significant association between the *GNB3* T allele and AD ($P = 0.049$), with no significant differences in the frequencies of *ADRB1* genotypes between cases and controls.

Since *ADRB1* and *GNB3* are functionally related, we tested if genetic interactions between them existed, by using either sample stratification or logistic regression models. Interestingly, in the stratified analysis we found that the *GNB3* TT genotype (OR = 4.00, 95% CI 1.65–9.71) and *GNB3* T allele (OR = 2.06, 95% CI 1.4–3.1, not shown) were clearly associated with increased risk for AD in individuals with *ADRB1* CC genotype, but not in those carrying *ADRB1* G alleles (Table 2). Conversely, *ADRB1* CC genotype was clearly associated with increased risk for AD in individuals bearing *GNB3* TT genotype (OR = 4.17, 95% CI 1.5–11.9, not shown). To analyze the strength of the interaction, we generated multivariate logistic regression models, with or without interaction terms included; the comparison of the model without or with interaction terms revealed a marginally significant ($P = 0.09$) interaction between *GNB3* and *ADRB1*. Furthermore, the analysis of the model including the interaction terms, summarized in Table 3, revealed that *GNB3* contributed significantly to the model after adjustment for age at onset, gender and ApoE genotype; the association with AD reached statistical significance for the individuals with the genotypes *ADRB1* CC and *GNB3* CT (OR = 3.48, 95% CI 1.27–9.58) or *ADRB1* CC and *GNB3* CT (OR = 2.07, 95% CI 1.01–4.26). In summary, both the stratification (Table 2)

Table 1
ADRB1 and *GNB3* genotype distribution

	ADRB1 genotype			Relative risk[a] (odds ratio)
	CC	CG	GG	C vs. G
AD	106 (0.61)	61 (0.35)	8 (0.05)	1.14
Control	97 (0.56)	68 (0.39)	8 (0.05)	ns
	GNB3 genotype			
	TT	CT	CC	T vs. C
AD	36 (0.21)	80 (0.46)	59 (0.33)	1.36 (1.00–1.84)
Control	27 (0.16)	71 (0.41)	75 (0.43)	0.049

Figures are numbers of individuals. Frequencies are shown in parenthesis.
[a] Relative risk with the 95% confidence interval (in parenthesis). The *P* value for the χ^2 test is shown; ns, non-significant.

Table 2
Crude analysis of the association between Alzheimer's disease and *GNB3* genotype by *ADRB1* genotype

ADRB1 genotype	*GNB3* genotype[a]			*GNB3* odds ratio[b]	
CC	CC	CT	TT	CT vs. CC	TT vs. CC
AD	32 (0.30)	50 (0.47)	24 (0.23)	1.88 (1.02–3.45)	4.00 (1.65–9.71)
Control	48 (0.49)	40 (0.41)	9 (0.09)		
Any G				CT vs. CC	TT vs. CC
AD	27 (0.39)	30 (0.43)	12 (0.17)	0.97 (0.47–2.01)	0.67 (0.27–1.65)
Control	27 (0.36)	31 (0.41)	18 (0.24)		

[a] Figures are numbers of individuals. Frequencies are shown in parenthesis.
[b] Relative risk with the 95% confidence interval (in parenthesis).

Table 3
Adjusted analysis of the association between Alzheimer's disease, *GNB3* and *ADRB1* genotypes

		GNB3 odds ratio[a]		
		CC	CT	TT
ADRB1	CC	1 (Ref)	2.07 (1.01–4.26)	3.48 (1.27–9.58)
Genotype	Any G	1.66 (0.73–3.77)	1.85 (0.84–4.06)	1.19 (0.45–3.14)

The model was adjusted for gender, age at onset and *APOE* genotype, and included interaction terms (P for interaction = 0.09).
[a] Relative risk with the 95% confidence interval (in parenthesis). Ref: group taken as the reference.

and the logistic regression approaches (Table 3) clearly indicated that to carry a particular genotypic combination of the *GNB3* and the *ADRB1* genes (*GNB3* TT or CT and *ADRB1* CC) was associated with risk for Alzheimer's disease.

Although association of the *GNB3* T allele [2,33] and the *ADRB1* C allele [1] with essential hypertension has been reported, the association of these alleles with AD appear to be independent on hypertension in our sample, since the effect was the same in normotensive and hypertensive individuals (not shown).

Since previous studies revealed differences in activity for the *ADRB1* and the *GNB3* polymorphisms [23,33],

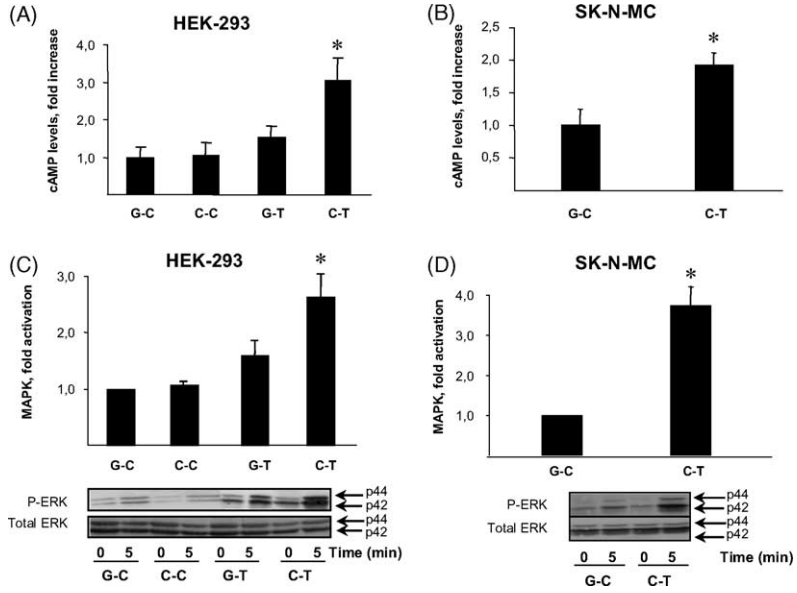

Fig. 1. β-Agonist dependent adenylyl cyclase (A, B) and MAPK (C, D) activity of HEK-293 cells (A, C) and SK-N-MC (B, D) transiently transfected with the indicated combinations of the *ADRB1* and *GNB3* allelic forms. Isoproterenol-induced cAMP levels, after normalization by forskolin-induced cAMP levels, were referred to the conditions chosen as control (*ADRB1* G/*GNB3* C transfected cells). MAPK activation was determined by immunodetection of activated-phosphorylated forms of ERK1/2, normalized by total MAPK levels as detailed in Section 2, and referred to the *ADRB1* G/*GNB3* C combination of allelic forms. Representative gels are shown below. Data presented in all panels are the mean with standard error (bars) of four/five independent experiments. Statistical significance of the differences was examined with the Student's *t*-test (* $P < 0.05$).

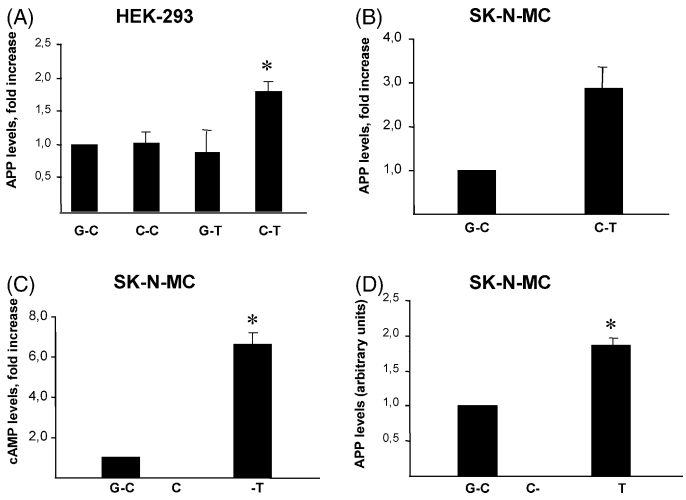

Fig. 2. Expression levels of APP in HEK-293 and SK-N-MC cells transfected with different combinations of *ADRB1* and *GNB3* allelic forms. The levels of APP in control conditions or upon stimulation with the β-agonist isoproterenol were determined by Western blotting as detailed in Section 2, in HEK-293 (A) or SK-N-MC neuroblastoma cells (B) transiently transfected with the indicated allelic combinations. After normalization by tubulin levels, data were referred to the conditions chosen as control (*ADRB1* G/*GNB3* C transfected cells). Data presented are the mean with standard error (bars) of three independent experiments. In (C) and (D), the basal levels of cAMP (C) and APP (D) were determined in SK-N-MC stably transfected with the indicated combinations of the *ADRB1* and *GNB3* allelic forms. After normalization by forskolin-induced cAMP levels (C) or tubulin expression levels (D), data were referred to the conditions chosen as control (*ADRB1* G/*GNB3* C transfected cells). Data are the mean with standard error (bars) of two independent experiments performed in triplicate. Statistical significance of the differences were examined with the Student's *t*-test ($^*P < 0.05$).

we examined the effect of combinations of *ADRB1* and *GNB3* alleles in adrenergic-induced intracellular cAMP production. The activity of the four possible combinations between *ADRB1* G/C and *GNB3* C/T was measured in the human embryonic kidney cell line HEK293, that lacks endogenous β1AR, and in the human neuroblastoma cell line SK-N-MC, transfected either stably or transiently with the corresponding allelic forms of both genes. As shown in Fig. 1A and B, isoproterenol-induced adenylyl-cyclase activity was significantly higher (between two and three-fold, depending on the cell type) in cells expressing the combination *ADRB1* C/*GNB3* T than in those expressing *ADRB1* G/*GNB3* C. This strongly suggested that the presence of the alleles conferring risk for AD (*ADRB1* C and *GNB3* T) could be associated with increased β-adrenergic responsiveness in neuronal cells in vivo. Consistent with an increased coupling to signaling pathways, this combination of polymorphisms also resulted in a two- to four-fold increased MAPK activation upon isoproterenol stimulation in both HEK-293 and SK-N-MC neuroblastoma cells (Fig. 1C and D).

To further search for functional consequences of the increased responsiveness of the *ADRB1* C/*GNB3* T combination, we explored its effects on the expression of APP. In agreement with previous reports showing that cAMP up regulates APP levels, we found that the cells transfected with the *ADRB1* C/*GNB3* T alleles displayed higher increases in APP protein upon isoproterenol treatment in both HEK-293 and SK-N-MC neuroblastoma cells (Fig. 2A and B). The same results were obtained at the mRNA level by using both semi-quantitative RT–PCR and cDNA microarrays (not shown). Moreover, basal APP protein expression was clearly increased in SK-N-MC cells stably expressing the *ADRB1* C/*GNB3* T alleles combination compared with those bearing *ADRB1* G/*GNB3* C alleles, consistent with a parallel increase in cAMP levels under these experimental conditions (Fig. 2C and D).

4. Discussion

The data presented here point to a functional interaction between the *ADRB1* and *GNB3* genes in the development of AD, and suggest that the AD risk associated with these genes directly correlates with an enhanced β1-adrenergic signaling that leads to increased cAMP cellular levels. The levels of cAMP have been found to be altered—either increased or decreased—in tissues of AD patients compared with those of controls. Data of our study are consistent with reports showing that cAMP levels are elevated in cerebral microvessels [10] and cerebrospinal fluid [21] from AD patients, and that cAMP appears to co-localize with amyloid deposits in the cerebral vessels of AD patients [22].

Head injury is the major non-genetic risk factor for AD [11,26,28], and head trauma can trigger deposition of Aβ in the brain [32]. Adrenergic activation occurring during major trauma [29] would promote Gs/adenylyl cyclase-mediated increases in intracellular cAMP levels, leading to enhanced cAMP-dependent protein kinase activity (PKA), which is the predominant mechanism by which this second messenger controls cell function. Our data in transfected HEK-293 and neuroblastoma cells suggest that such response would

be significantly increased (approximately two- to four-fold) in individuals carrying the particular combination of *ADRB1* C and *GNB3* T alleles. An increased coupling of the β_1-adrenergic transduction machinery to the MAPK pathway can also be detected in cells expressing the *ADRB1* C/*GNB3 T* combination. Moreover, our study clearly indicates that the increase in APP protein levels in response to adrenergic agonists is clearly enhanced in cells expressing such allelic combination. It has been reported that cAMP regulates APP synthesis and secretion: in neuronal cells, cAMP regulates APP amyloidogenic proteolysis [13]; in astrocytes, cAMP modulates both APP gene transcription [15] and protein proteolysis [16] and, consequently, an elevation of cAMP produces an intracellular accumulation of cell-associated APP holoprotein, containing amyloidogenic peptides [16]. Furthermore, we have found by using expression microarray analysis of human neuroblastoma cells, that adrenergic agonists and cAMP inducers increase the APP mRNA levels, most probably by transcriptional mechanisms (manuscript in preparation).

All these studies establish potential links between adrenergic activation, cAMP and amyloidogenesis, considered a crucial event in AD. On the other hand, it has been reported that increases in cAMP levels can also lead to enhanced expression of *APOE* [9] and to an elevated phosphorylation of tau [12,36]. Therefore, alterations of cAMP could be involved in AD pathogenesis at multiple levels.

The absence of Mendelian inheritance indicates that late-onset AD is a complex disease in which genetic and non-genetic factors are likely to be interacting. The ε4 allele of apolipoprotein E gene (*APOE* ε4) has been associated with an increased risk of developing AD [6]. However, many AD cases have no *APOE* ε4 alleles. Thus, it is likely that there are additional AD risk factors, both genetic and environmental, still to be identified. Genetic association is a powerful tool to investigate genes involved in complex diseases [14,31], specially when it is supported by the functional analysis of the allelic variants [8], as that performed in this report. Genome screens have implicated regions of several chromosomes, including chromosomes 12 and 10 (for recent review, see [18,34]). The region of chromosome 12 linked with AD has received considerable attention [7,30] and, although three genes that lie within this region (LRP, A2M and LBP-1c) have been associated with risk for AD, it is tempting to add the *GNB3* gene, mapped to 12pter-p12.3 [19], as a candidate gene for the susceptibility locus on chromosome 12. Interestingly, the *ADRB1* gene lies (10q24–q26) within the linkage region of another susceptibility locus for late onset AD that has been proposed in chromosome 10 (for review see [18]). The association of the combination of the *GNB3* T and *ADRB1* C alleles with increased risk for AD could be an example of the interaction between genetic and environmental factors in triggering late-onset Alzheimer, and open novel therapeutic strategies for this disease.

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