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Evidence Supporting a Role for the Calcium-Sensing Receptor in Alzheimer Disease

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Abstract

The calcium-sensing receptor (CASR) is a G-protein coupled, transmembrane receptor that responds to changes in Ca^{2+} levels. We hypothesized that the CASR could have a role in Alzheimer disease (AD) given expression of the CASR in brain, knowledge that calcium dysregulation promotes susceptibility to neuronal cell damage, the important role that the CASR plays in calcium regulation, and the fact that systemic calcium homeostasis and G-protein signal transduction are altered in AD patients. To investigate the association of CASR variation in AD susceptibility, we genotyped a polymorphic dinucleotide repeat marker within intron 4, one SNP within the promoter region and three non-synonymous SNPs within exon 7 of the CASR gene and tested for association analysis, using a well-characterized cohort of AD cases ($n = 692$) and controls ($n = 435$). The dinucleotide repeat polymorphism was significantly associated with AD status (OR = 1.62; 95% CI: 1.27–2.07, $P = 0.00037$, Bonferroni corrected $P = 0.0011$) and the three non-synonymous SNP haplotype was borderline associated with AD status ($P = 0.032$, Bonferroni corrected $P = 0.096$). Stratifying by APOE4 allele carrier status revealed that the significant association was only in non-APOE4 carriers (OR of 1.90; 95% CI: 1.37–2.62, $P = 0.0001$). We also investigated whether apoE or β amyloid could activate the calcium-sensing receptor. The receptor activation assays revealed that apoE as well as β amyloid activated the CASR and that the level of activation appeared to be isoform dependent for apoE. These data support our hypothesis that the CASR has a role in AD susceptibility, particularly in individuals without an APOE4 allele.

Keywords

CASR; calcium dysregulation; AD; APOE

INTRODUCTION

Alzheimer disease (AD) is a complex neurodegenerative disease. Several genes have been implicated in the etiology of AD. Mutations in the amyloid precursor protein (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2) genes have been identified as causal in

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early onset familial forms of AD [Selkoe and Podlisny, 2002]. The apolipoprotein E (APOE) gene has been implicated in susceptibility to late onset AD however the at risk allele, APOE4, is neither necessary nor sufficient for the development of AD [Selkoe and Podlisny, 2002]. Additional genetic modifiers for AD susceptibility are likely to exist. The disruption of calcium homeostasis is an alternative hypothesis of AD etiology or a significant mechanism in the pathological cascade of the disease. There are many reviews of the calcium hypothesis for AD and neurodegeneration [Khachaturian, 1994; Bojarski et al., 2008; Mattson, 2007; Stutzmann, 2007; Thibault et al., 2007; Toescu and Verkhatsky, 2007a,b]. Support for this hypothesis include the observation that calcium ion (Ca^{2+}) levels outside of the normal physiologic range result in neuronal cell death [Verkhatsky and Toescu, 1998] and the Ca-mediated signaling system is altered in the aging nervous system resulting in altered neuronal functioning and/or cell death [Khachaturian, 1991]. Additional support for calcium's involvement in AD pathogenesis stems from the pivotal role that calcium ions play in normal neuronal function. For instance, Ca^{2+} regulates synaptic plasticity [Kater et al., 1989], increases in intracellular Ca^{2+} result in prevention of microtubule and microfilament assembly in neurons [Mattson et al., 1993], and the presence of elevated extracellular Ca^{2+} facilitates more rapid expression of neuronal injury than when extracellular Ca^{2+} levels are normal [Farber, 1981]. Ca^{2+} levels are known to modulate neurotransmitter release and neuronal membrane excitability [Khachaturian, 1991] and Ca^{2+} participates in neuronal gene regulation [West et al., 2001]. Calcium dysregulation is further implicated in AD since each of the genes currently known to influence AD susceptibility so far (APP, PSEN1, PSEN2, and APOE) affects intracellular Ca^{2+} levels and/or calcium signaling [Misra et al., 2001; LaFerla, 2002; Mattson, 2002]. Since Ca^{2+} concentration plays such an important role in normal neuronal function, abnormal Ca^{2+} levels and calcium signaling produce symptoms of brain aging, neuronal degeneration and death, then mechanisms responsible for maintaining Ca^{2+} levels may contribute to AD susceptibility, including the calcium sensing receptor (CASR).

The CASR is a G-protein coupled, transmembrane receptor primarily involved in systemic calcium homeostasis. The CASR is expressed in tissues involved in calcium homeostasis such as the parathyroid and kidney, however, the CASR is also expressed in tissues where its function is less readily apparent, such as the brain [Brown, 1999]. Examples of known functions of the CASR are: (1) regulating serum Ca^{2+} levels and responding to Ca^{2+} levels by regulating parathyroid hormone (PTH) levels; (2) regulating urinary calcium excretion; and (3) conversion of vitamin D to its active metabolite, 1, 25 dihydroxyvitamin D. A significant number of AD patients demonstrate altered systemic calcium homeostasis in relation to normal age-matched controls, including a decrease in serum calcium level, increase in serum PTH level, increase in urinary calcium excretion, and a decrease in serum 1, 25 dihydroxyvitamin D concentration [Ogihara et al., 1990; Landfield et al., 1992; Sato et al., 1998]. Additionally, the level of inositol triphosphate (IP_3), phospholipase C production and protein kinase C activation are altered in the Alzheimer brain [Kurumatani et al., 1998] and the signal transduction cascade initiated by activation of the CASR involves these substances. The level of CASR activation may directly affect signal transduction, mobilization of intracellular Ca^{2+} stores, and subsequent calcium channel activation in brain tissue, disrupting local Ca^{2+} homeostasis.

To test the hypothesis that the CASR is involved in AD susceptibility, we performed an association study involving a dinucleotide repeat polymorphic marker within intron 4, three nonsynonymous SNPs in exon 7 thought to impact systemic calcium regulation, and one promoter SNP, using a well-characterized cohort of AD patients and non-demented controls. We also conducted in vitro receptor activation studies of the CASR using β amyloid and apolipoprotein isoforms to determine if these might activate the receptor, using a sensitive reporter gene assay.

MATERIALS AND METHODS

Subjects

The case cohort (n = 692) was obtained from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC). These individuals were diagnosed either clinically (n = 640) or by autopsy (n = 52) with late onset sporadic AD. The average age of onset for the subjects was 72.5 ± 6.1 years; 70% were female and all subjects were Caucasian. The clinical diagnosis was made at a Consensus Conference after extensive evaluation of subjects based on the NINCDS/ADRDA criteria [McKhann et al., 1984]. A detailed description of the clinical evaluations that were conducted for the case and controls subjects has been described in a previous study that utilized these same subjects [Wang et al., 2002]. The control cohort (n = 435) was obtained from the same geographical region as the cases. The average age of the controls at time of last assessment was 76.6 ± 5.7 years. They were all Caucasian and 61% were female.

Genomic DNA was extracted from whole blood leukocytes or brain tissue collected at autopsy using the methods and reagents from the Qiaamp kit (Qiagen, Valencia, CA). All cases and controls were obtained using protocols approved by the University of Pittsburgh Institutional Review Board.

Genotyping

Dinucleotide repeat—Intron four of the CASR gene contains a variable CA repeat polymorphism that was chosen to increase variability within the haplotype block that would not be possible with the addition of another SNP. The primers and PCR cycling conditions developed by Tsukamoto et al. [1998] were utilized with the exception that the forward primer was fluorescently labeled in order to detect the products on the ABI377. We amplified all cases and controls for the polymorphism, electrophoresed the products and conducted analysis of fragment size using the ABI377 and ABI genotyper software (Applied Biosystems Inc., Foster City, CA).

SNPs—Exon seven of the CASR gene contains three non-synonymous SNPs, which together with the intron four dinucleotide represents the second of three haplotype blocks within the CASR gene [Yun et al., 2007]. A986S, R990G, and Q1011E (rs1801725, rs1042636, and rs1801726 respectively) are in close proximity to one another, which made the use of some genotyping techniques impossible and for this reason these three SNPs were genotyped via sequencing. One 347bp PCR product that encompassed all three SNPs was generated using the following primers and annealing temperature 5'-GACCCTCCACAGCAGCAACG-3' (F), 5'-TGACAAAGCTCTGTGAACTGG-3' (R), and 51°C. The reverse primer was utilized for sequencing. Sequencing data were generated (Rexagen Corporation, Seattle, WA) and analyzed using Sequencher software (Gene Codes Corporation, Ann Arbor, MI).

rs9883099 is located 8bp upstream of a vitamin D response element in the promoter and represents the first of three haplotype blocks within the CASR gene. This SNP was chosen to represent this haplotype block because of its proximity to the vitamin D response element. This SNP was genotyped using RFLP/PCR. The primers, annealing temperatures and restriction endonuclease for genotyping were 5'-GCACGGGAGAGGGCAGGAGA-3' (F), 5'-GTCCCGGTTCCCTTACCGTC-3' (R), 62°C and NlaIV. APOE genotypes were determined using RFLP/PCR as previously described [Kamboh et al., 1995].

Statistical Analyses of Genotype Data

Analysis of dinucleotide repeat and promoter SNP data

The dinucleotide repeat was analyzed as a dichotomous variable with small (≤ 228 bp) versus large (≥ 230 bp) alleles and analyzed using chi-square tests for significance and calculation of odds ratios using a 2×2 contingency table approach. Difference in age at onset based on genotype for the dichotomized data was evaluated using Kaplan–Meier curves and log rank tests to assess the equality of the survival distributions. The allele and genotype frequencies for rs9883099 were analyzed using chi-square tests for significance and calculation of odds ratios using a 2×2 contingency table approach.

Linkage disequilibrium (LD) and haplotype analyses

H-clust, a program for selecting tagging SNPs based on LD [<http://www.wpic.pitt.edu/WPICCompGen/hclust.htm>] [Rinaldo et al., 2005], was utilized to determine if the SNP in the promoter (rs9883099) and the three SNPs in exon seven (rs1801725, rs1042636, rs1801726) were in strong LD or if they would be chosen as tagging SNPs. Linkage disequilibrium was < 0.80 for any of the SNPs. Subsequently, haplotypes were generated for the three SNPs in exon seven (non-synonymous SNP haplotype) to capture (untyped) genetic variation at the locus and/or possible interactions among these SNPs. Haplotypes were generated using haplo.stats in the R package for statistical computing [<http://www.r-project.org>]. Differences in haplotype frequencies between cases and controls were tested using the T5 statistic [Zhao et al., 2000]. To test for significant association, permutation testing was utilized by randomly assigning case or control status to each individual, performing 1,000 permutations and calculating the T5 statistic for each of the permuted data sets to calculate an empirical P -value.

Diplotype analyses

After correcting for multiple testing there was a borderline association with the three non-synonymous SNP haplotype and because of previous reports in the literature suggesting that these SNPs are associated with serum Ca^{2+} levels, additional exploratory analyses were pursued to consider possible mechanism. The QQ genotype for Q1101E and the AA genotype for A986S are associated with lower Ca^{2+} levels and the RR genotype of R990G associated with higher Ca^{2+} levels [Cole et al., 2001; Scillitani et al., 2004]. We therefore designated “low serum Ca^{2+} diplotypes” to be “AA GG QQ” or “AA GR QQ and examined whether the frequency of individuals with multiple “low serum Ca^{2+} genotypes” was increased among individuals with AD.

Analysis of the relationship between APOE and CASR in AD

To potentially inform mechanism and given the significant association of AD with APOE4, we investigated whether the CASR polymorphisms were an independent risk factor for AD. Analyses were conducted independently using the dichotomized data for the dinucleotide repeat as well as the three SNP haplotype and diplotype data in exon seven of the CASR. APOE data were dichotomized into APOE4 carriers and APOE4 non-carriers. Analyses were conducted using logistic regression.

Expression Studies

Transfection—Cos-1 cells (ATCC, Manassas, VA) were cotransfected with the wild type CASR construct, PKC luciferase reporter gene construct and *Renilla* control construct (pRL-SV40 from Promega, Madison, WI) using the Transfectam[®] system (Promega). The CASR construct was cloned into the pcDNA1.1 Amp vector (Invitrogen, Carlsbad, CA). The PKC luciferase reporter gene construct consisted of a protein kinase C responsive promoter linked

to a firefly luciferase reporter gene. The control construct constitutively produces renilla luciferin protein in Cos-1 cells. The control protein was used as an internal control to normalize the activity of the luciferase reporter, to minimize experimental variability due to variation in cell viability or transfection efficiency from culture to culture. The cultures were incubated for 48 hr in DMEM with 10% FBS.

Exposures—DMEM was removed and replaced by SMEM for 6 hr prior to exposures to reduce the amount of luciferin protein that potentially accumulated due to chronic calcium exposure from the DMEM that would activate the CASR. The half-life of the luciferin protein in mammalian cells is 3 hr. Six separate cultures were set up for each exposure and control. Separate exposures were set up for apoE2, apoE3, apoE4 (Intracell Corp, Rockville, MD) and A β _{1–42} (Sigma, St. Louis, MO) to a final concentration of 25 μ M. The exposure time was 5 min, then the reactions were washed and lysed. The control reactions had no exposure to apoE2, apoE3, apoE4 and A β _{1–42}. They did, however, have exposure to FBS, which contains various proteins such as albumin.

Luciferase assay—The Dual-Luciferase reporter assay system (Promega) and a luminometer (Turner Design, Sunnyvale, CA) were utilized to quantitate the amount of firefly and *Renilla* luciferase activity. The *Renilla* luciferase activity served as an internal control for cell viability and transfection efficiency. All firefly luciferase activities were normalized using the *Renilla* luciferase activities. The mean values from the six independent cultures for apoE2, apoE3, apoE4 and A β _{1–42} were compared to the control using a paired *t*-test where a $P < 0.05$ was considered significant.

RESULTS

Intron 4 Dinucleotide Repeat Results

The CASR intron 4 dinucleotide repeat polymorphism was significantly associated with AD status ($P = 0.00037$, Table I). Using a Bonferroni correction a significant P -value would be $0.05/3 = 0.017$ (three association tests performed were the dinucleotide polymorphism, the promoter SNP and the exon seven haplotype data) and therefore we conclude that we have a significant association with this polymorphism with a corrected $P = 0.0011$. The dichotomous data indicated that carriers of the short allele were at increased risk of getting AD compared to non-carriers, with an odds ratio (and C.I.) of 1.62 (1.27–2.07) (Table I). There was no relationship between CASR genotype and age of onset of AD based on dichotomized data ($P = 0.399$).

SNP Results

The promoter SNP was not significantly associated with AD status (Table I). The non-synonymous SNPs were trending toward being associated with AD status when analyzed as haplotypes ($P = 0.032$, Bonferroni corrected $P = 0.096$; Table II). Interestingly, the “AGQ” haplotype for the non-synonymous SNPs was almost twice as frequent among AD cases as among controls (frequency = 0.040 vs. 0.073, respectively). We next tested whether the frequency of individuals with either “AA GG QQ” or “AA GR QQ” diplotypes (that is “low serum Ca²⁺ diplotypes”) differed by AD status. The frequency of “low serum Ca²⁺ diplotypes” was significantly higher among cases (11.1%, 63/569) versus controls (6.3%, 20/318), OR = 1.86 (95% CI: 1.1–3.1, $P = 0.019$).

CASR and APOE Results

CASR genotype was significantly associated with AD status among individuals who are non-APOE4 allele carriers after correcting for multiple testing, but did not appear to significantly influence risk among individuals who carried an APOE4 allele. Carriers of the

dichotomized “small allele” for the intron 4 dinucleotide repeat who were E3/E3 homozygotes had an increased risk of AD ($P = 0.0001$, Bonferroni corrected $P = 0.0002$; Table III) over those who were not a carrier of the “small allele” with an odds ratio of 1.90 (95% CI: 1.37–2.62). This was not the case for carriers of the “small allele” who had at least one E4 allele (E3/E4 and E4/E4). APOE4 carrier status was not relevant to the association between the non-synonymous SNP haplotype for CASR and AD status, most likely due to the number of haplotypes. However, there was a borderline significance for the frequency of “low serum Ca^{2+} diplotypes” by AD status. Among those with the E3/E3 genotype, the “low serum Ca^{2+} diplotype” was higher ($P = 0.051$) among cases than controls (OR = 1.98; 95% CI: 0.99–3.97), whereas there was no relationship among E4 carriers (OR = 1.69; 95% CI: 0.58–4.94, $P = 0.33$).

ApoE and β Amyloid Receptor Activation Results

The calcium-sensing receptor demonstrated increased receptor activity by β amyloid ($\text{A}\beta_{1-42}$) as well as apoE (Fig. 1). $\text{A}\beta_{1-42}$ activated the receptor by 36% ($P < 0.001$), apoE3 activated the receptor by 49% ($P < 0.01$), and apoE4 activated the receptor by 39% ($P < 0.001$). ApoE2 did not significantly activate the receptor, although the findings for apoE2 are trending towards significance ($P = 0.06$).

DISCUSSION

Our hypothesis that the CASR contributes to the susceptibility threshold for AD is consistent with the association and receptor activation data we present. We found a genetic association for the CASR with AD status and, interestingly, this association appeared to be limited to individuals without an APOE4 allele. Further, the activation of the CASR by β amyloid and apoE provides a potential functional link to explain the association. The role that the CASR plays in AD susceptibility may be explained through a mechanism related to local ionized calcium regulation in the brain, systemic Ca^{2+} regulation, endogenous receptor activators other than Ca^{2+} , or that activation of the CASR initiates a signal transduction cascade whose downstream activities initiate processes important to AD pathogenesis.

The function of the CASR in the brain is not well understood compared to its function in systemic Ca^{2+} regulation; however it is possible that the CASR provides localized Ca^{2+} regulation in the brain. Systemic Ca^{2+} regulation via the CASR involves PTH; however PTH would likely not be involved in localized brain Ca^{2+} regulation. Evidence supporting Ca^{2+} regulation via the CASR independent of PTH secretion [Kos et al., 2003] makes localized Ca^{2+} regulation in the brain via the CASR a putative mechanism for the association of the CASR with AD status. Additionally, evidence continues to build in support for a role of the CASR in brain cell differentiation including a role for Ca^{2+} impacting cellular proliferation of oligodendrocyte progenitor cells acting through the CASR [Chattopadhyay et al., 2008].

Strengthening the hypothesis that the CASR is involved with AD susceptibility is the fact that systemic issues related to calcium homeostasis are noted in AD patients. As mentioned previously AD patients demonstrate altered systemic calcium homeostasis, including altered serum Ca^{2+} , PTH and 1,25-dihydroxyvitamin D levels when compared to normal age matched controls. [Ogihara et al., 1990; Landfield et al., 1992; Sato et al., 1998]. The non-synonymous SNPs within the CASR that were investigated have functional consequences and have been implicated in serum Ca^{2+} levels with the QQ genotype for Q1101E and the AA genotype for A986S associated with lower Ca^{2+} levels and the RR genotype of R990G associated with higher Ca^{2+} levels [Cole et al., 2001; Scillitani et al., 2004; Vezzoli et al., 2007]. Although the difference in frequency of each of these genotypes separately is not statistically significantly in AD cases, we did find that the haplotype (or diplotype)

containing the “low serum Ca^{2+} alleles (or genotypes)” was higher among AD cases than controls.

Activation of the CASR (whether Ca^{2+} , apoE, β amyloid or another of the many activators of this receptor) in the brain would elicit a signal transduction cascade that resulted in down stream release of intracellular Ca^{2+} , IP_3 , phospholipase C and protein kinase C. Each of these downstream products has been found to be altered in AD patients [Kurumatani et al., 1998]. These products have the potential to elicit signal transduction cascades in addition to activating calcium channels, which could further amplify the detrimental effects of CASR activation.

The fact that apoE and β amyloid activate the CASR may in part explain the mechanisms by which these substances influence neurodegeneration. β amyloid and apoE affect intracellular and extracellular Ca^{2+} levels [Mattson et al., 1992; Wang and Gruenstein, 1997; Ye et al., 1997; Tolar et al., 1999]. β amyloid destabilizes calcium homeostasis [Mattson et al., 1992] and activates the CASR [Mattson et al., 1992; Ye et al., 1997]. Additionally, calcium appears to play a role in initiating β amyloid aggregation and amyloid fibrils in vitro [Isaacs et al., 2006]. These findings support the theory that at least one mechanism by which β amyloid is involved with neurodegeneration involves disruption of Ca^{2+} levels. Experiments with apoE have demonstrated an influx of calcium as well as an increase in intracellular calcium via a G-protein, protein kinase C pathway [Wang and Gruenstein, 1997; Tolar et al., 1999], that the intracellular Ca^{2+} levels induced neuronal death [Tolar et al., 1999], and that calcium-associated cell death resulted when neuronal cells were treated with apoE4 in addition to calcium homeostasis being disrupted [Veinbergs et al., 2002]. We provide preliminary evidence that the above association between apoE and calcium may be through activation of the CASR, influencing neuronal Ca^{2+} levels, and potentially leading to disruption of local calcium homeostasis that could result in the neuronal cells being more vulnerable to damage.

Our data indicate that the CASR may play a more prominent role in susceptibility to AD among individuals that lack an APOE4 allele. The dinucleotide data support this hypothesis, and the low serum Ca^{2+} diplotype analyses are consistent with it, but the haplotype analyses were not supportive. This discrepancy may be due to issues related to the low number of individuals for different haplotypes and diplotypes after breaking them into APOE4 carriers and non-carriers.

Why might the CASR may be more influential for AD susceptibility among non-APOE4 carriers? ApoE4 is hypothesized to promote β amyloid deposition [Schmechel et al., 1993; Nagy et al., 1995; Pirttila et al., 1997] and as previously mentioned calcium appears to be important to β amyloid aggregation and the formation of amyloid fibrils [Isaacs et al., 2006]. One possibility, therefore, is that apoE4 and calcium dysregulation promote the deposition of β amyloid independently and in the absence of the burden of apoE4, calcium dysregulation becomes a prominent issue for AD susceptibility.

This is the first study to investigate the CASR in relation to AD and supports a role for the CASR in AD susceptibility. The data also add to the body of evidence supporting the calcium hypothesis for AD pathogenesis. Additional studies are needed to determine if the intronic dinucleotide repeat polymorphism itself or another variant in LD with the polymorphism is important in the susceptibility to AD and the non-synonymous SNP haplotypes require more investigation to determine if a particular haplotype is indeed associated with AD susceptibility. Such will require a larger replicate population.

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FIG. 1.

CASR activation by A β_{1-42} and apolipoprotein E isoforms. P-values are based on comparison to control values. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I

Results of CASR Intron 4 and Promoter Polymorphisms

Polymorphism	Genotypes N (%)			P-value	OR (95% CI)
	LL	LS	SS		
Dichotomized repeat					
Cases	352 (51.02)	244 (35.36)	94 (13.62)		
Controls	273 (62.76)	110 (25.29)	52 (11.95)	0.00037	1.62 (1.27–2.07)
Genotypes N (%)					
Polymorphism	AA	AC	CC	P-value	
Promoter (rs9883099)					
Cases	262 (39.8)	299 (45.4)	97 (14.8)		
Controls	151 (36.9)	184 (45.0)	74 (18.1)		0.348

TABLE II
Frequency of CASR Nonsynonymous SNP Haplotype in Cases and Controls

Haplotype	SNP		Frequency ($P = 0.032$)	
	Q1011E	A986S	G990R	Cases
1	E	A	G	0.000
2	E	S	R	0.000
3	E	A	R	0.045
4	Q	S	G	0.000
5	Q	A	G	0.073
6	Q	S	R	0.181
7	Q	A	R	0.701

TABLE III

Relationship of AD Status by Dinucleotide Repeat Small Allele Carriers and Non-Carriers Stratified by apoE4 Carrier Status

<u>Small dinucleotide repeat allele</u>				
Approach	Carrier	Non-carrier	P-value	OR (95% CI)
Non-E4 carriers				
Case	156 (52.7)	140 (47.3)		
Control	114 (37.0)	194 (63.0)	0.0001	1.90 (1.37–2.62)
<u>“Low” Ca++ diplotype</u>				
Approach	Carrier	Non-carrier	P-value	OR (95% CI)
Non-E4 carriers				
Case	25 (10.5)	212 (89.5)		
Control	13 (5.6)	218 (94.4)	0.051	1.98 (0.99–3.97)
<u>Small dinucleotide repeat allele</u>				
Approach	Carrier	Non-carrier	P-value	OR (95% CI)
E4 carriers				
Case	182 (46.2)	212 (53.8)		
Control	35 (42.2)	48 (57.8)	0.50	1.18 (0.73–1.90)
<u>“Low” Ca++ diplotype</u>				
Approach	Carrier	Non-carrier	P-value	OR (95% CI)
E4 carriers				
Case	38 (11.5)	292 (88.5)		
Control	4 (7.1)	52 (92.9)	0.33	1.69 (0.58–4.94)