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Glucocorticoid-related genetic susceptibility for Alzheimer's disease

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Because glucocorticoid excess increases neuronal vulnerability, genetic variations in the glucocorticoid system may be related to the risk for Alzheimer's disease (AD). We analyzed single-nucleotide polymorphisms in 10 glucocorticoid-related genes in a population of 814 AD patients and unrelated control subjects. Set-association analysis revealed that a rare haplotype in the 5′ regulatory region of the gene encoding 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) was associated with a 6-fold increased risk for sporadic AD. Results of a reporter-gene assay indicated that the rare risk-associated haplotype altered *HSD11B1* transcription. HSD11B1 controls tissue levels of biologically active glucocorticoids and thereby influences neuronal vulnerability. Our results indicate that a functional variation in the glucocorticoid system increases the risk for AD, which may have important implications for the diagnosis and treatment of this disease.

INTRODUCTION

Alzheimer's disease (AD) is the major neurodegenerative disorder of the elderly, and is characterized by progressive cognitive deficits such as impairment of memory. Histopathologically, AD is characterized by extracellular β-amyloid plaques, intraneuronal neurofibrillary tangles, synaptic loss and neuronal death. Hippocampal neurons are among the first cells to degenerate in the brain of patients affected by AD (1). The hippocampus is a brain region important for memory (2) and contains a high density of glucocorticoid receptors (3,4). Glucocorticoids are known to influence cognitive functions (5–9) and high concentrations of these steroid hormones have adverse effects on neurons, especially in the hippocampus, and reduce neuronal ability to survive a variety of coincident insults, including AD-associated neurotoxicity (10–12). Therefore, increased circulating hydrocortisone concentrations, which are consistently found in AD (9,13–16), may play a role in the pathophysiology of this disease. The possibility that the glucocorticoid system may be implicated in AD led us to hypothesize that polymorphisms in genes involved in the regulation of the glucocorticoid system may influence the risk for this disease.

In a case–control study in two independent, ethnically distinct populations, we investigated single nucleotide polymorphisms (SNPs) in genes coding for the following proteins: corticotropin-releasing hormone (*CRH*), corticotropin-releasing hormone binding protein (*CRHBP*), receptor for the adrenocorticotropic hormone (*MC2R*), 11β-hydroxysteroid dehydrogenase type 1 and 2 (*HSD11B1*, *HSD11B2*), glucocorticoid receptor (*NR3C1*), glucocorticoid modulatory element binding protein 1 and 2 (*GMEB1*, *GMEB2*), steroidogenic factor 1 (*NR5A1*) and nuclear receptor coactivator 2 (*NCOA2*).

For statistical analysis, we used the set-association method (17) which evaluates accurately several sets of polymorphic markers. To date, case–control association studies mostly follow a candidate-gene approach by examining one gene at a time. Although powerful, this approach ignores the genetic complexity of sporadic AD, which is caused by variations in several genes, each with a small effect on overall disease risk. Moreover, this marker-by-marker procedure causes severe inflation of the type I statistical error (i.e. false positive results)

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due to multiple testing in one population and overlooks possible interactions between susceptibility genes. By simultaneous analysis of several polymorphic markers, the set-association method controls for type I statistical error and considers the existence of gene–gene interactions.

RESULTS

Genotyping of 11 SNPs in glucocorticoid-related genes (Table 1) and of the gene encoding apolipoprotein E (*APOE*) in 814 individuals revealed that two genes, *APOE* and *HSD11B1*, were associated with AD. The level of significance reached by these two genes was equal to 0.002 (Fig. 1A). The final corrected significance P_{min} was 0.006. Family history for AD or dementia did not influence the results. Separate set-association analysis in the Swiss and Mediterranean samples revealed similar results, i.e. *APOE* and *HSD11B1* contributed to AD risk (Fig. 1B and C). In the Mediterranean sample ($n = 423$), the gene encoding the adrenocorticotropic hormone receptor (*MC2R*) was also associated with the disease (Fig. 1C). Set-association analysis excluding APOE confirmed that, among the examined glucocorticoid-related genes, only *HSD11B1* is a susceptibility gene for AD (Fig. 1D). Conventional χ^2 analysis also revealed significant over-representation ($P = 0.008$) of the rare allele *A* of *HSD11B1* (rs846911) in AD patients (2.9%) as compared with control subjects (0.5%). This difference corresponded to an odds ratio (OR) of 6.2 [95% confidence interval (CI): 1.4–28.4]. The *APOE4* allele was also more frequent in AD patients (48.0%) than in control subjects (25.4%, $P < 0.000001$), corresponding to an OR of 3.0 (95% CI: 2.1–3.5). In addition to rs846911, which is located 2037 bp upstream of the start codon of *HSD11B1*, we also analyzed rs860185, which is located 718 bp upstream of the start codon, and observed that both SNPs had identical allelic frequencies and were in complete linkage disequilibrium (LD). Thus, recombination events in the 5′ regulatory region of *HSD11B1*, at least up to 2 kb upstream of the start codon, were absent in 814 individuals. This region is characterized by two extended haplotypes spanning 1.3 kb, the rare and disease-associated haplotype *A-T* and the frequent haplotype *C-A* (Fig. 2).

To exclude the possibility that the observed association is due to LD of the *HSD11B1* haplotype with SNPs in the 5′ and 3′ adjacent genes, we genotyped part of our population for SNPs rs713073 and rs926346, located in *CAMK1G* (encoding calcium/calmodulin-dependent protein kinase 1G) and *IRF6* (encoding interferon regulatory factor 6), respectively, and failed to observe a significant association of either SNP with AD ($P \geq 0.15$, Fig. 2). To further exclude the possibility of LD with yet not identified SNPs in *HSD11B1*, we sequenced in 24 chromosomes the six exons of *HSD11B1*, the intron–exon boundaries and 2.1 kb upstream the start codon. Eight of the 24 chromosomes contained the disease-associated haplotype. We found no evidence for additional coding SNPs or those influencing RNA splicing. Sequencing of 2.1 kb upstream the start codon revealed the absence of SNPs other than rs846911 and rs860185.

Taken together, these results suggest that the haplotype spanning SNPs rs846911 and rs860185 is directly associated with the risk for AD. To determine the effects of this haplotype on transcriptional regulation, we cloned haplotype-specific promoter fragments into the pGL3-Basic vector. Promoter activity was assayed using a dual-luciferase system in human embryonic kidney cells (HEK-293). The risk-associated, rare haplotype reduced luciferase activity by 20% relative to the common haplotype ($P < 0.000001$), suggesting that the effects of the rare haplotype on AD risk are related to reduced *HSD11B1* transcription (Fig. 3).

DISCUSSION

The results of the present study revealed that a rare haplotype in the 5′ regulatory region of the gene encoding HSD11B1 was associated with increased risk for sporadic AD. We did not find evidence for another causal SNP in the 5′ and 3′ adjacent genes, the six exons, the intron–exon boundaries or 2.1 kb upstream the start codon of *HSD11B1*. Moreover, we did not find strong evidence for association of other glucocorticoid-related genes with AD. Because we investigated one SNP per gene on average, however, the possibility still exists that unlinked causal genetic loci could have been missed. In addition, extensive sequencing of the broader 5′ region of *HSD11B1* beyond the already sequenced 2.1 kb should follow to define the exact haplotype borders and to detect possible additional SNPs associated with AD. Thus, the negative results presented herein do not justify definite exclusion of specific glucocorticoid-related genomic regions as significant susceptibility factors for AD.

We also found evidence for a functional relevance of the rare, disease-associated haplotype. In particular, the results of a reporter-gene assay indicated that the disease-associated haplotype reduced *HSD11B1* transcription. HSD11B1 is an intracellular enzyme which catalyses the interconversion of glucocorticoids and thereby controls tissue levels of biologically active glucocorticoids. While in cell culture HSD11B1 seems to act predominantly as a reductase (18), this enzyme favors dehydrogenase activity in cell homogenates, where cells are damaged, resulting in inactivation of biologically active glucocorticoids (19–21). Moreover, an *in vivo* study has shown potent dehydrogenase activity of HSD11B1 in the rat hippocampus (22). In the present study, carriers of the rare haplotype with reduced *HSD11B1* transcription may therefore show less inactivation of hydrocortisone resulting in increased neuronal vulnerability to AD-associated neurotoxicity and, finally, in increased risk for the clinical manifestation of AD. Our results further suggest that increased circulating hydrocortisone concentrations, which are consistently found in AD (9,13–16) may play a role in the pathophysiology of this disease.

In summary, we report first evidence for a genetic association between the glucocorticoid system and AD. Importantly, results of a reporter-gene assay indicate that the rare risk-associated haplotype alters *HSD11B1* transcription. HSD11B1 controls tissue levels of biologically active glucocorticoids which are known to influence neuronal vulnerability (10–12). Owing to the strong effect on the risk for AD, this haplotype may have a potential value as a diagnostic marker with high positive predictive value. Because this is the first study reporting an association between a glucocorticoid-related gene and AD,

Table 1. Genotype distributions of SNPs included in the final step of set-association analysis. Two SNPs (rs915032 in *NR5A1* and rs877181 in *NCOA2*) were not considered due to significant deviations from Hardy–Weinberg equilibrium ($P < 0.05$) in control subjects

SNP	Swiss sample		Mediterranean sample		Total sample	
	HCS ($n = 287$)	AD ($n = 104$)	HCS ($n = 176$)	AD ($n = 247$)	HCS ($n = 463$)	AD ($n = 351$)
rs6195 (NR3C1)						
AA	93.9%	95.2%	93.9%	95.2%	93.9%	95.2%
AG	6.1%	4.8%	6.1%	4.8%	6.1%	4.8%
GG	—	—	—	—	—	—
rs2318376 (GMEB1)						
AA	34.6%	37.4%	43.5%	40.6%	38.2%	39.7%
AG	45.2%	38.4%	41.7%	46.3%	43.8%	44.0%
GG	20.2%	24.2%	14.8%	13.1%	18.0%	16.3%
rs914563 (GMEB2)						
CC	95.1%	94.2%	95.7%	94.8%	95.4%	94.6%
CT	4.9%	5.8%	3.7%	5.2%	4.4%	5.4%
TT	—	—	0.6%	—	0.2%	—
rs2272725 (NCOA2)						
GG	87.1%	89.4%	88.0%	86.3%	87.5%	87.2%
GC	12.5%	10.6%	12.0%	13.3%	12.3%	12.5%
CC	0.4%	—	—	0.4%	0.2%	0.3%
rs948322 (MC2R)						
AA	11.8%	9.7%	14.3%	7.2%	12.7%	8.0%
AG	45.2%	50.5%	44.7%	45.8%	45.1%	47.2%
GG	43.0%	39.8%	41.0%	47.0%	42.2%	44.8%
rs6159 (CRH)						
AA	77.4%	78.6%	74.2%	80.2%	76.2%	79.8%
AC	21.4%	19.4%	23.3%	18.0%	22.1%	18.5%
CC	1.2%	1.9%	2.5%	1.6%	1.7%	1.7%
rs32897 (CRHBP)						
GG	4.1%	—	1.8%	4.9%	3.2%	3.5%
GA	24.2%	29.3%	27.0%	25.5%	25.3%	26.6%
AA	71.7%	70.7%	71.2%	69.5%	71.5%	69.9%
rs846911 (HSD11B1)						
AA	—	—	—	—	—	—
AC	—	1.0%	1.2%	3.6%	0.5%	2.9%
CC	100%	99.0%	98.8%	96.4%	99.5%	97.1%
rs5479 (HSD11B2)						
CC	92.2%	94.1%	89.6%	86.3%	91.2%	88.6%
CA	7.8%	5.9%	10.4%	13.3%	8.8%	11.1%
AA	—	—	—	0.4%	—	0.3%

SNP, single-nucleotide polymorphism according to dbSNP; HCS, healthy control subjects; AD, patients with Alzheimer's disease; gene names are given in parentheses.

independent replications will be crucial to corroborate the results, which may have important implications for therapeutic approaches aimed at preventing neurodegeneration in AD.

MATERIALS AND METHODS

Population

Genetic studies were conducted in two independent populations: a Swiss sample (391 participants) and a Mediterranean sample (423 participants from Italy and Northern Greece). Patients were recruited from the outpatient memory clinics of the participating institutions. The clinical diagnoses of AD ($n = 351$) were made according to the NINCDS-ADRDA criteria, and were based on medical interview, physical examination, neuropsychological testing, brain MRI or CT, as well as blood and CSF tests. The mean onset age (±standard deviation) of AD was 67 ± 9 years, the mean Mini-mental State Examination (MMSE) score was 20 ± 6. There were 211 (60%) females among the AD patients. Family history information was obtained by direct interview of the patients and their care-givers on the occurrence of AD, dementia and signs of memory loss in the first degree relatives of the patients and was positive for 49.3% of the participants. The control group ($n = 463$) consisted of elderly individuals without severe medical conditions who were either the spouses of AD patients or subjects recruited from the outpatient clinics of the participating institutions. Dementia and memory deficits in control subjects were excluded by neuropsychological testing, consisting of the CERAD neuropsychological test battery and the MMSE. The mean age was 68 ± 9 years, the mean MMSE score was 29 ± 1. There were 245 (53%) females among the control subjects. Informed consent was obtained from all participants, and the local human studies committees approved the study protocol.

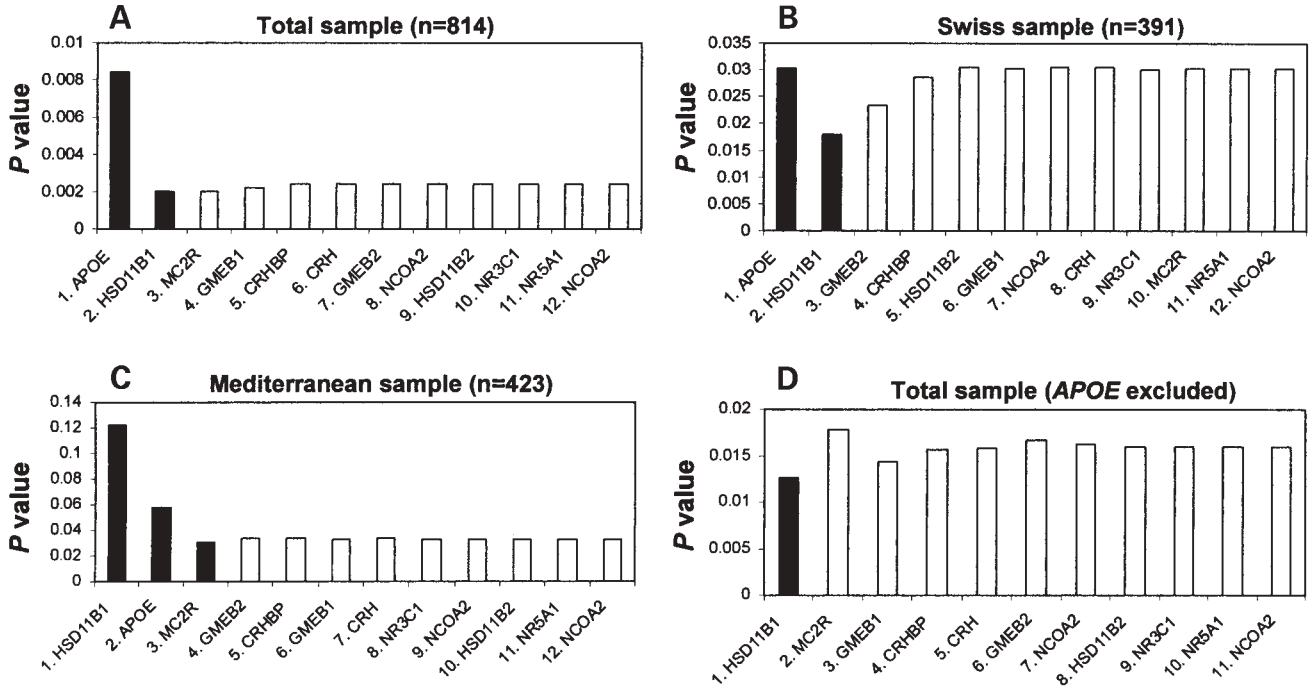

Figure 1. Significance level *P* as a function of SNPs in glucocorticoid-related genes and in *APOE*. SNPs were added to the model stepwise according to their contribution to the disease risk. SNPs decreasing *P*-value to a minimum (black bars) exert a significant effect. White bars indicate SNPs not contributing to the disease risk significantly, i.e. *P*-value is increasing due to introduction of statistical noise. (**A**) total sample ($n = 814$); (**B**) Swiss sample ($n = 391$); (**C**) Mediterranean sample ($n = 423$); (**D**) total sample excluding *APOE*.

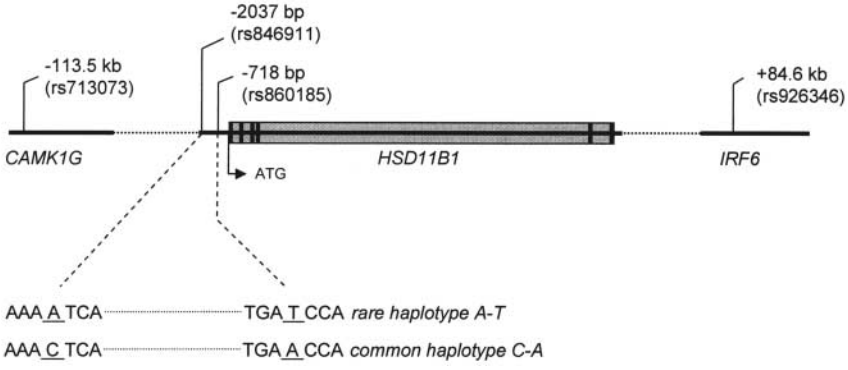

Figure 2. Schematic representation of the *HSD11B1* region and of the analyzed SNPs. All distances are given relative to the *HSD11B1* start codon, rs numbers represent the reference sequence numbers according to the dbSNP. *HSD11B1* is shown in gray with short vertical lines representing the exons. *CAMK1G* and *IRF6* are the 5′ and 3′ adjacent genes to *HSD11B1*, respectively.

Genotyping

Information on polymorphic sites was derived from the database of single nucleotide polymorphisms (dbSNP) established by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/SNP/index.html). Twenty-one SNP candidates in 10 genes were selected for genotyping. Of these SNP candidates, eleven were polymorphic in 100 chromosomes (rs915032 in *NR5A1*, rs6195 in *NR3C1*, rs2318376 in *GMEB1*, rs914563 in *GMEB2*, rs2272725 and rs877181 in *NCOA2*, rs948322 in *MC2R*, rs6159 in *CRH*, rs32897 in *CRHBP*, rs846911 in *HSD11B1*, and rs5479 in *HSD11B2*) and were genotyped in the study sample by the Masscode™ system (23). APOE genotyping was done on the LightCyclerTM (24).

Statistics

We used the SUMSTAT program (http://linkage.rockefeller.edu/ott/sumstat.html), which has been developed for the statistical analysis with the set-association method (17). The set-association method combines the information derived from measurements of allelic association and deviation from Hardy–Weinberg equilibrium into a single, genome-wide statistic. SNPs with high Hardy–Weinberg disequilibrium

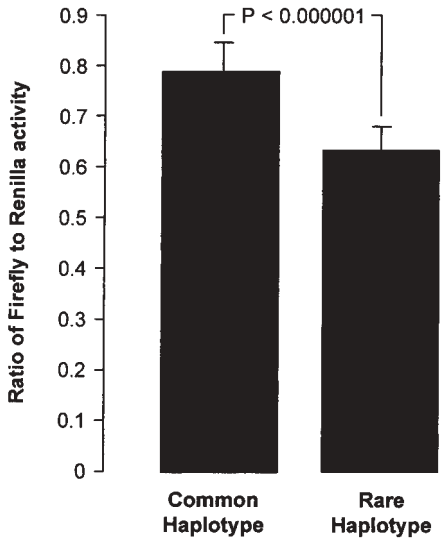

Figure 3. Reporter-gene assay (dual-luciferase) of the *HSD11B1* regulatory region with two haplotype-specific constructs in HEK-293 cells. Columns indicate the mean of 20 independent experiments, error bars were plotted as SD. Statistical analysis was done by a two-sided Student's *t*-test. $P < 0.000001$.

(HWD) values in the control population are set equal to zero ('trimming') and are not considered for further statistical analysis. For the remaining SNPs, effects of allelic association with disease and HWD values are combined into one statistic, because moderate HWD values in affected individuals are indicative of genetic association to a susceptibility locus (17). Genome-wide significance of this statistic is calculated by permutation tests. In the present study, two SNPs with high HWD values ($\chi^2 > 3.9$, $P < 0.05$) in the control population were trimmed. The number of permutation tests was set at 50 000.

Reporter-gene assay

A 2.1 kb long fragment of the human *HSD11B1* regulatory region (accession number: NT_021877), containing the common haplotype (C-A), was generated by gene synthesis (Medigenomix, Martiensried, Germany), cloned into the BlueScript vector (Stratagene) and subsequently into the promoter-less pGL3-Basic vector (Promega). A site-directed mutagenesis kit (Stratagene) was used to generate the construct containing the rare haplotype (A-T). Several DNA clones containing the A-T haplotype were selected. All constructs were verified by sequence analysis.

Human embryonic kidney cells (HEK, 293) were cultured in DMEM (Life Technologies) supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. Transfection of 90% confluent cultures, grown in 24-well plates, was performed with lipofectamine 2000 (Invitrogene), using 1.5 μg DNA per well. *Firefly* luciferase expressing constructs containing the common or rare haplotype in the human *HSD11B1* regulatory region were co-transfected in a molar ratio of 150:1 with pRL-TK *renilla* luciferase vector (Promega) as the internal control for transfection efficiency. Twenty-four hours later, cells were harvested with the passive lysis buffer of the Dual-Luciferase system (Promega) and the luciferase activities were measured according to the manufacturer's protocol. After deduction of background values (luciferase activity of the promoterless PGL3 vector), the ratio of *firefly* to *renilla* activities was calculated and the average were built for each group. Experiments were repeated in 20 independent wells.

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