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Is the urea cycle involved in Alzheimer's disease?

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Abstract

Since previous observations indicated that the urea cycle may have a role in the Alzheimer's disease (AD) process, we set out to quantify the expression of each gene involved in the urea cycle in control and AD brains and establish whether these genes could be genetic determinants of AD. We first confirmed that all the urea cycle enzyme genes are expressed in the AD brain. The expression of arginase 2 was greater in the AD brain than in the control brain. The presence of the rare arginase 2 allele rs742869 was associated with an increase in the risk of AD in men and with an earlier age at onset for both genders. None of the other genes in the pathway appeared to be differentially expressed in the AD brain or act as genetic determinants of the disease.

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Keywords

Alzheimer's disease; urea cycle; citrulline NO cycle; ammonium; nitric oxide; polyamines; arginase; association study

Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly, accounting for 50–70% of cases. Given the continuing increase in life expectancy, the number of subjects suffering from this devastating neurodegenerative disorder will undoubtedly increase in the coming years. AD is characterized by the accumulation of extracellular β -amyloid ($A\beta$) deposits (generated by the proteolytic cleavage of amyloid precursor protein) in senile plaques and by the formation of neurofibrillary tangles inside neurons [1;2]. It is known that AD results from the interaction of both environmental and genetic factors. Several genes have been clearly identified as determinants of the disease. Mutations in three different genes encoding the amyloid precursor protein, presenilin 1 and presenilin 2 have been linked to hereditary, early-onset forms of AD (although the latter account for less than 1% of the total number of cases) [3]. Beyond these rare, monogenic forms of AD, the $\epsilon 4$ allele of Apolipoprotein E gene (APOE) has been recognized as a major genetic determinant for the more common, late-onset forms of AD [4]. Most recently, genome wide association (GWA) studies have revealed that three other genes (CLU, CR1 and PICALM) are associated with the risk of developing late-onset AD [5; 6]. However, these GWA studies also indicate that additional genetic susceptibility remains to be identified.

Our laboratory recently performed a microarray study in order to identify genes that are differentially expressed in AD and control brains [7]. We identified an induction of expression of the gene for ornithine transcarbamylase (OTC, an enzyme involved in the urea cycle) in the AD brain (Figure 1). This initial transcriptomic observation was supported by the finding that OTC expression was restricted to vascular endothelial cells in the AD brain. Furthermore, OTC activity in the cerebrospinal fluid (CSF) was almost 9-fold higher in probable AD cases than in controls. We subsequently reported that the OTC gene could be a minor genetic determinant of AD [8]. This result was unexpected, since the urea cycle occurs almost exclusively in the liver, where it enables the conversion of ammonium (NH_3) into urea and thus subsequent elimination of the latter by the kidneys [9]. Interestingly, we observed that the other enzymes in the urea cycle (also called the ornithine cycle) were also expressed in the AD brain. As a consequence, these observations indicated the potential involvement of the urea cycle in the pathophysiological process in AD. In order to evaluate this hypothesis, we first quantified the level of expression of four genes coding for enzymes directly involved in the urea cycle (ASS, ASL, Arginase 1 and Arginase 2) and two genes controlling ammoniac intake into this cycle (GS and CPS1). We then sought to establish whether or not any of these genes are genetic determinants of AD.

Materials and methods

mRNA quantification

Alzheimer's disease brains were obtained at autopsy from 114 early- and late-onset sporadic AD patients accessioned from the Greater Manchester region of the United Kingdom during the period 1986–2001 (mean $\pm$ standard deviation age at death: 72.3 ± 9.7 ; mean age at onset: 65.9 ± 10.3 ; 51% male) [10]. All patients were of Caucasian ethnic origin. Pathological diagnoses were made in accordance with the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) Neuropathological Criteria. All patients were at Braak stage 5 or 6 at the time of death. Control brains were obtained from an initial set of 167 brains recruited

from routine autopsies carried out at the Hospices Civils de Strasbourg (Strasbourg, France) (mean $\pm$ standard deviation age at death: 79.0 ± 6.1) [11]. Again, all control subjects were Caucasian. Recruitment was designed to exclude cases of dementia; individuals were recruited from a general hospital rather than medical institutions in which the majority of patients presented with dementia. Most subjects were brought to the hospital less than 48 hours before death by the emergency services and were living at home prior to admission. Cases referred to autopsy for neurological diseases (again according to the CERAD Neuropathological Criteria) were excluded.

Total RNA was extracted from frozen frontal cortex brain tissue from the 114 AD and 167 control samples using a phenol/chloroform protocol (Trizol® reagent, Invitrogen) [7]. The quality of total RNA was assessed using an Agilent 2100 bioanalyzer and the ratio of ribosomal RNA 28S/18S was systematically estimated using Agilent BioSizing software. Total RNA samples from 37 controls (age at death: 80.1 ± 6.2 , 42% male, braak stage <2) and 38 AD cases (age at death: 74.7 ± 9.0 , 39% male) were randomly selected for quantification of the expression of the urea cycle genes (0.6 μ g per assay) and the housekeeping β -actin gene (0.1 μ g per assay), according to the supplier's instruction (Quantigene®, Panomics) [10].

Case-control studies and genotyping

The Lille case-control study [10]—The AD and control subjects were all Caucasians (Table 1). A diagnosis of probable AD was established according to the Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised, (DSM-III-R) and National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria. Controls were recruited from retirement homes or from electoral rolls. All were free of DSM-III-R dementia and had intact cognitive function and a MMSE score above 25. Participation was voluntary and each subject or his/her next of kin provided informed consent. Control subjects with a family history of dementia were excluded.

The Rouen case-control study [12]—All subjects were Caucasians originating mainly from western France (Table 1). A clinical diagnosis of probable AD was established according to the DSM-III-R and NINCDS-ADRDA criteria. Control subjects (mainly the patients' spouses) had to have a MMSE score above 28.

The Birmingham case-control study [12]—The UK AD and control subjects were all Caucasians from Greater Birmingham and Manchester (Table 1). A clinical diagnosis of probable AD was established according to the DSM-III-R and NINCDS-ADRDA criteria. Age at disease onset was not available for these probable AD cases. Control subjects were confirmed to be non-demented by either use of the DSM-III-R questionnaire or a MMSE score over 28.

The Pittsburgh case-control study [10]—The USA AD and control subjects were all Caucasians from the Pittsburgh area (Table 1). All patients were recruited from the University of Pittsburgh Alzheimer's Disease Research Center. Clinical diagnoses were made according to the NINCDS/ADRDA criteria. The Alzheimer's Disease Research Center follows a standard evaluation protocol, including examination of the personal and family medical history, general medical and neurological examinations, a psychiatric interview, neuropsychological testing and a magnetic resonance imaging scan. Control subjects were recruited from the same Western Pennsylvania region as the AD patients and were determined to be non-demented following a detailed clinical examination (i.e. an MMSE score above 28).

Genotyping of all studied single-nucleotide polymorphisms (SNPs) was performed by the Genoscreen company (Lille, France) using TaqMan technology. The genotyping protocols are available on request.

Statistical analyses

All statistical analyses were performed with SAS software, release 8.02 (SAS Institute, Cary, NC, USA). Comparison of the amounts of mRNA for AD cases and controls was performed using a non-parametric Wilcoxon test. An analysis of covariance using a general linear model was also used for this comparison after (i) log-transformation and normalization of the mRNA data and (ii) adjusted for mRNA degradation according to the 28S/18S ribosomal RNA ratio. However, the results were not changed by this additional analysis and so are not shown here.

A univariate comparison of genotype and allele distributions in AD cases and controls analysis was performed using Pearson's χ^2 test. Review Manager software (release 5.0) was used to estimate the overall effect as a Mantel-Haenszel fixed odds ratio (OR). In order to adjust for age and *APOE* $\epsilon 4$ genotype, we also analyzed the association of the rs742869 SNP with the AD risk in men and women by using an additive logistic regression model and a recessive model. The co-dominant model is equivalent to an allelic association approach when the conditions for Hardy-Weinberg equilibrium are met [13], as was indeed the case in a meta-analysis of the overall population. Before performing pooled analyses, we tested for inter-population homogeneity using the Breslow-Day statistic [14]. The association between the rs742869 polymorphism and age at onset was assessed using a mixed model adjusted for gender and using the center as a random variable.

Results

We compared the expression levels of 6 genes involved in the urea cycle (GS, CPS1, ASS, ASL, Arg1 and Arg2) in 37 control brains and 38 AD brains by directly quantifying the amounts of mRNA (see the Materials and Methods section) (Figure 2). In confirmation of our previous report [7], all 6 genes appeared to be expressed in AD and control brains, even though the Arg1 gene exhibited a low expression level in comparison with the 5 others. Only the Arg2 gene appeared to be more highly expressed in AD brains than in control brains (+55%, $p=0.01$).

In parallel with this transcriptomic analysis, we looked at whether any of the genes might have an impact on the risk of developing AD. We selected 86 tag-SNPs characterized by an $r^2 < 0.8$ and a minor allele frequency $> 10\%$ in the HapMap database (Supplementary table S1). Eighty-one SNPs were then successfully genotyped in two independent "discovery" sub-samples (487 French subjects and 364 American subjects) randomly selected from two large case-control studies from these populations (as described in the Materials and Methods section). We compared genotype distributions for AD cases and controls in the whole, combined "discovery" sample, with and without stratification for gender (Supplementary table S1). This stratification was mainly prompted by the fact (i) that the OTC gene (the only gene identified as a genetic determinant of the AD in this pathway) is located on chromosome X and (ii) of significant statistical interactions between some of the SNPs analyzed, gender and AD risk (data not shown and for instance $p=0.002$ for rs742869). According to our analytical design, only differences in genotype distribution presenting a p value below $p=0.0003$ were considered to be statistically significant after Bonferroni correction ($p=0.05$ divided by 81 and then by two for gender stratification, when applied). According to this statistical criterion, only the genotype distribution of the rs742869 (A/G) SNP (located within intron 4 of the Arg2 gene) appeared to be significantly different in men ($p=0.0002$) but not in women ($p=0.94$). This SNP was not in linkage disequilibrium (LD) with the other SNPs located in the Arg2 gene (Supplementary figure S1).

We next extended the analysis of the intronic rs742869 SNP to the complete Lille case-control study (n=2191) and to two other independent case-control samples from France (Rouen, n=1170) and the UK (Birmingham, n=541). The association of the rare G allele with an increase in AD in men was confirmed in the combined sample (Table 2 and Figure 3). Using a recessive model, we observed that the rare GG genotype was associated with an increase in the AD risk (OR=1.76 (95% CI [1.27–2.43]; p=0.0006; adjusted for age, APOE status and center). In agreement with our previous results, we did not find any association of this SNP with the risk of AD in women. We searched for a potential association of rs742869 in publicly available databases on GWASs in AD. No information was available in Li and Reiman GWASs [15; 16] since this SNP was neither studied nor in LD with any of the tag-SNPs studied in these studies (according to the HapMap database, data not shown). Rs742869 was not reported to be associated with the risk of developing AD at p<0.001 in the Harold et al. GWAS [5]. However, it is possible that this SNP could be nominally associated between 0.001 and 0.05. Similarly, since no data were available in terms of gender stratification, it was not possible to assess a specific association of this SNP with AD risk in males.

We searched for an association between the rs742869 SNP and age at onset (Table 3). We observed that the rare G allele was associated with an earlier age at onset in the study population as a whole (p=0.05). In agreement with our previous results, men carrying the GG genotype had a significantly earlier age at onset than AA carriers (p=0.037). This association was not observed in women (p=0.22).

Lastly, even though the results are not statistically significant due to the low number of AD brains analyzed, we also noticed that individuals bearing at least one rs742869 minor G allele presented lower Arg2 expression (Supplementary table S2) than AA carriers did (–26%, p=0.17), with a more pronounced decrease in men (–35%, p=0.11).

Discussion

In the present work, we confirmed that (i) all the genes encoding for enzymes involved in the urea cycle are expressed in the brain of AD cases [7] and (ii) Arg2 is the major arginase isoform in the human frontal cortex [17]. Interestingly, our observations are consistent with a recent expression analysis of the genes coding for the urea cycle enzymes in human brain stem cells [18]. Furthermore, we found that the Arg2 gene expression was higher in AD brains than in control brains.

Remarkably, our genetic approaches revealed a potential association of the Arg2 gene with the risk of developing AD. Although we analyzed 81 Tag-SNPs within 6 urea cycle genes, the only observed association was that of an Arg2 SNP with both AD risk and earlier age at onset in males from a sample of 2806 AD cases and 1416 controls. In addition, we showed a trend for a decrease in Arg2 expression in the brain of AD cases bearing the rs742869 minor allele. These data suggest that either rs742869 or another SNP in LD may be involved in the control of Arg2 expression. Since this decrease appeared to be potentially more pronounced in men, we may postulate that the systematic gender-effects we observed, could be linked to a sex-dependent difference in binding or expression of transcription factors as reported in numerous cases [19] Using Genomatix software, we observed that the presence of a rs742869 SNP minor allele creates a binding site for the microphthalmia-associated transcription factor (MITF). The involvement of MITF has never been documented in Alzheimer's disease and its putative role in genotype-and/or sex-dependent expression of Arg2 is unclear. Similarly, we may also postulate that either rs742869 or another SNP in LD could alter splicing events in a sex dependent manner, as it has been shown recently for the low-density lipoprotein receptor [20]. Hence, an in-depth sequence analysis and further functional analyses will be required to

clearly identify the link between the Arg2 SNPs, the expression of the Arg2 enzyme and the risk of AD.

When taken together with our previous studies on OTC [7;8], the present work suggests that both OTC and Arg2 play a role in the AD process. At this stage, it is still not clear whether an induction of the urea cycle would be protective or harmful. However, the fact that the rs742869 G minor allele is associated (in men) with an increase in AD risk, an earlier age at onset and a potential decrease in Arg2 expression may indicate that the urea cycle induction is protective. These observations are supported by literature reports in which arginase expression was associated with neuroprotection and arginase deficiency was thought to favor neurodegeneration [21–23].

Given this context, one can legitimately hypothesize that the induction of OTC expression and the increase in Arg2 expression may constitute the brain's attempt to maintain a low level of NH₃. This could be compromised in the AD brain by an increase in NH₃ production following increase in protein degradation on one hand and a decrease in GS activity (as previously described in the brain of AD cases) on the other hand [24]. Remarkably, the similarity between clinical and pathological features in hyperammonemic disorders and AD suggests the involvement of hyperammonemia in AD itself [25–27]. Accordingly, induction of OTC expression could be a response to the failure of NH₃ detoxification; an increase in Arg2 expression in the AD brain may facilitate the OTC-dependent elimination of NH₃.

One can also hypothesize that Arg2 is involved in the AD process because of its central role in the switch between arginine metabolism, NO production (via the citrulline NO cycle) and synthesis of polyamines (see Figure 1). First, arginase expression leads to a decrease in NO production through consumption of the arginine pool and the resulting decrease in iNOS expression [28–30]. Interestingly, NO is known to have neuroprotective effects at low concentrations and neurotoxic (and potentially neurodegenerative) effects at excessively high concentrations [21]. In such a context, an increase in Arg2 expression may help to limit NO production and thus protect the brain from neurodegeneration. Secondly, an increase in Arg2 expression may lead to increased polyamine synthesis via ODC, since Arg2 activity is the limiting factor for production of these compounds in neurons [29;31]. Interestingly, polyamines are involved in the maintenance of adult neural stem cells [32] and display protective effects in neurodegenerative models [33;34]. The significance of this hypothesis is reinforced by the fact that neuronal expression of ODC increases in proportion to the disease stage - indicating that the urea cycle may play a role in the AD process [35].

In conclusion, our results confirm and emphasize the potential role of the urea cycle in the AD process and show that this pathway might be involved in a neuroprotective mechanism that has yet to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic representation of the urea cycle and its connected metabolism

Urea cycle is associated with the citrulline NO pathway (in bold lines) and connected to the polyamines synthesis. (ASL: Argininosuccinate Lyase; ASS: Argininosuccinate synthase; CPS1: Carbamylphosphate synthase; GS: Glutamine Synthase; NOS: NO synthase; ODC: Ornithine decarboxylase; OTC: Ornithine transcarbamylase.)

Figure 2. Relative expression of Glutamine Synthase and each gene of the urea cycle in cortex of 37 controls (white) and 38 AD (grey) subjects
Intensity of the luminescent signal for each gene of interest was normalized with the intensity of the signal for β -actin.

Figure 3. Association of the rare allele of rs742869 with the risk of AD in four independent populations for men, women or whatever the gender

Table 1

Characteristics of the populations used for this study.

Populations		n	Age at start of the study (mean±SD)	Age Onset (mean±SD)	Gender distribution (%age Males)
North of France (Lille)	Controls	604	73.0 ± 8.1	/	36%
	AD Cases	1587	74.1 ± 8.8	69.6 ± 8.2	33%
West of France (Rouen)	Controls	502	68.8 ± 9.0	/	45%
	AD Cases	668	69.9 ± 9.1	65.4 ± 9.2	39%
United Kingdom (Birmingham)	Controls	155	68.4 ± 10.5	/	36%
	AD Cases	386	75.1 ± 6.9	NA	42%
USA (Pittsburgh)	Controls	155	74.3 ± 5.8	/	48%
	AD Cases	165	77.0 ± 6.1	73.0 ± 6.1	53%

Alleles and genotypes distributions of rs742869 SNP are indicated for each subpopulation and combined population for men, women or whatever the gender.

Table 2

Men only		Alleles distribution n(freq)		Genotypes distribution n(freq)			p
Population	n	A	G	AA	AG	GG	
Lille (France)	Control	215	275 (0.64)	155 (0.36)	85 (0.40)	105 (0.49)	25 (0.12)
	AD cases	518	618 (0.60)	418 (0.40)	190 (0.37)	238 (0.46)	90 (0.17)
Rouen (France)	Control	226	271 (0.60)	181 (0.40)	81 (0.36)	109 (0.48)	36 (0.16)
	AD cases	257	292 (0.57)	222 (0.43)	86 (0.33)	120 (0.47)	51 (0.20)
Birmingham (UK)	Control	56	74 (0.66)	38 (0.34)	23 (0.41)	28 (0.50)	5 (0.09)
	AD cases	162	176 (0.54)	148 (0.46)	52 (0.32)	72 (0.44)	38 (0.23)
Pittsburgh (USA)	Control	75	93 (0.62)	57 (0.38)	23 (0.31)	47 (0.63)	5 (0.07)
	AD cases	87	90 (0.52)	84 (0.48)	24 (0.28)	42 (0.48)	21 (0.24)
Combined	Control	572	713 (0.62)	431 (0.38)	212 (0.37)	289 (0.51)	71 (0.12)
	AD cases	1024	1176 (0.57)	872 (0.43)	352 (0.34)	472 (0.46)	200 (0.20)
Women only		Alleles distribution n(freq)		Genotypes distribution n(freq)			p
Population	n	A	G	AA	AG	GG	
Lille (France)	Control	384	464 (0.60)	304 (0.40)	141 (0.37)	182 (0.47)	61 (0.16)
	AD cases	1030	1213 (0.59)	847 (0.41)	354 (0.34)	505 (0.49)	171 (0.17)
Rouen (France)	Control	272	317 (0.58)	227 (0.42)	94 (0.35)	129 (0.47)	49 (0.18)
	AD cases	408	485 (0.59)	331 (0.41)	142 (0.35)	201 (0.49)	65 (0.16)
Birmingham (UK)	Control	99	118 (0.60)	80 (0.40)	36 (0.36)	46 (0.46)	17 (0.17)
	AD cases	224	273 (0.61)	175 (0.39)	85 (0.38)	103 (0.46)	36 (0.16)
Pittsburgh (USA)	Control	80	94 (0.59)	66 (0.41)	22 (0.28)	50 (0.63)	8 (0.10)
	AD cases	78	101 (0.65)	55 (0.35)	31 (0.40)	39 (0.50)	8 (0.10)
Combined	Control	835	993 (0.59)	677 (0.41)	293 (0.35)	407 (0.49)	135 (0.16)
	AD cases	1740	2072 (0.60)	1408 (0.40)	612 (0.35)	848 (0.49)	280 (0.16)

Men + Women		Alleles distribution n(freq)			Genotypes distribution n(freq)			p
Population	n	A	G	AA	AG	GG		
Lille (France)	Control	744 (0.62)	464 (0.38)	227 (0.38)	290 (0.48)	87 (0.14)	0.29	
	AD cases	1874 (0.59)	1300 (0.41)	555 (0.35)	764 (0.48)	268 (0.17)		
Rouen (France)	Control	592 (0.59)	412 (0.41)	176 (0.35)	240 (0.48)	86 (0.17)	0.94	
	AD cases	779 (0.58)	557 (0.42)	228 (0.34)	323 (0.48)	117 (0.18)		
Birmingham (UK)	Control	192 (0.62)	118 (0.38)	59 (0.38)	74 (0.48)	22 (0.14)	0.29	
	AD cases	449 (0.58)	323 (0.42)	137 (0.35)	175 (0.45)	74 (0.19)		
Pittsburgh (USA)	Control	187 (0.60)	123 (0.40)	45 (0.29)	97 (0.63)	13 (0.08)	0.02	
	AD cases	191 (0.58)	139 (0.42)	55 (0.33)	81 (0.49)	29 (0.18)		
Combined	Control	1715 (0.61)	1117 (0.39)	507 (0.36)	701 (0.50)	208 (0.15)	0.08	
	AD cases	3293 (0.59)	2319 (0.41)	975 (0.35)	1343 (0.48)	488 (0.17)		

Table 3

Association of rs742869 with age at onset of the AD for codominant, dominant and recessive genetic model.

Genotypes	M+W		M		W	
	Age at Onset	p value	Age at Onset	p value	Age at Onset	p value
AA	69.1±8.7		68.4±8.1		69.5±9.0	
AG	68.5±8.7	0.052	67.8±8.5	0.119	68.9±8.8	0.408
GG	67.8±8.8		66.7±8.7		68.6±8.8	
AA	69.1±8.7	0.049	68.4±8.1	0.125	69.5±9.0	0.214
AG+GG	68.3±8.7		67.5±8.6		68.8±8.8	
AA+AG	68.8±8.7	0.045	68.0±8.3	0.068	69.2±8.9	0.382
GG	67.8±8.8		66.7±8.7		68.6±8.8	