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Neuroglobin and Alzheimer's dementia: Genetic association and gene expression changes

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Abstract

We previously reported strong genetic linkage on chromosome 14q to Alzheimer's Disease (AD) using the presence of co-morbid hallucinations as a covariate. Those results suggested the presence of a gene increasing the risk for a genetically homogeneous form of AD characterized by the absence of comorbid hallucinations. Here we report our follow up of that study through the analysis of single nucleotide polymorphisms (SNPs) in five functional candidate genes. This work provides significant evidence of association for the gene coding for Neuroglobin (*NGB*), a nervous system globin known to protect cells against amyloid toxicity and to attenuate the AD phenotype of transgenic mice. On further experiments we found that *NGB* expression is reduced with increasing age and lower in women consistent with their increased risk. *NGB* expression is up-regulated in the temporal lobe of AD patients consistent with a response to the disease process, as reported for *NGB* and hypoxia. We speculate that a compromised response due to DNA variation might increase the risk for AD. Our and others' data strongly support the involvement of *NGB* in AD.

Keywords

Neuroglobin; Alzheimer's disease; dementia; gene expression

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by deterioration of memory, cognition, behavior, emotion, and intellect. With the exception of rare early-onset forms with Mendelian inheritance it most commonly affects people over the age of 65. It is a major public health problem, affecting over 5 million Americans today, a number that could range from 11 to 16 million by the year 2050 (Alzheimer's Association data 2008; www.alz.org). Early-onset forms of AD (about 5% of cases) have an exclusively genetic etiology, with an autosomal dominant mode of inheritance and three identified causative genes

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PSEN1, *PSEN2* and *APP* (Goate, et al., 1991, Levy-Lahad, et al., 1995, Sherrington, et al., 1995). In late-onset AD variation in the apolipoprotein E (*APOE*) gene has been shown to be a genetic risk factor (Strittmatter, et al., 1993) but it is believed that many more remain to be identified (Daw, et al., 2000, Jarvik, et al., 1996). A number of pathogenic mechanisms have been implicated in the neurodegenerative process of AD, among the most studied being apoptosis, (Bamberger and Landreth, 2002, Shimohama, 2000, Takuma, et al., 2005) oxidative stress, (Cecchi, et al., 2002, Gibson and Huang, 2005, Nunomura, et al., 2006, Reynolds, et al., 2007, Shi and Gibson, 2007, Zhu, et al., 2007) hypoxia, (L. Li, et al., 2007, Peers, et al., 2007) and inflammation (McGeer, et al., 2006, Weisman, et al., 2006, Wyss-Coray, 2006). Although those mechanisms are closely related to each other, the exact cause – effect relationship that leads to disease remains unknown.

In an effort to find the remaining genetic loci contributing to late-onset AD risk, several linkage studies have been performed (Blacker, et al., 2003, Kehoe, et al., 1999, Pericak-Vance, et al., 2000) and have reported a large number of chromosomal regions potentially harboring risk loci. Although some of these regions appear more consistently in the literature (Bertram and Tanzi, 2004) no gene has been implicated with a certainty similar to that for *APOE*. In fact, recent genome scans for association have shown that it is unlikely for another locus to exist in the genome bearing a single risk variant with the effect size of *APOE* (Waring and Rosenberg, 2008). The lack of consistency and the weak results in both linkage and association studies likely reflect an underlying genetic and allelic heterogeneity. In a previous study we addressed heterogeneity through the incorporation of covariates in a genome wide linkage analysis and detected strong linkage with a LOD score of 3.91 on chromosome 14q when the presence or absence of hallucinations was considered (Avramopoulos, et al., 2005). Sequencing of multiple patients excluded the presence of mutations in *PSEN1*, which is located in the same region (Avramopoulos, et al., 2005). Here we report on a follow-up study of genetic association for selected candidate genes in the 14q region that provides significant evidence for the involvement of the neuroglobin gene (*NGB*), and we further show that *NGB* has an RNA expression profile that supports its involvement in AD. Our data, in combination with the previous functional studies, make *NGB* a very interesting candidate as a genetic determinant of AD risk.

2. Materials and Methods

2.1 Sample Description

Genotyping sample—We initially screened 5 candidate genes in our linkage region using a study design that attempted to reduce heterogeneity by integrating information on the presence of hallucinations. We genotyped 99 patients with comorbid hallucinations, 125 patients without hallucinations and 152 cognitively healthy control subjects aged 58 to 99 years (Table 1). Cases were from the NIMH collection and were assessed for psychotic symptoms as described (Avramopoulos, et al., 2005) while controls were from the collection of the Indiana cell repository (NCRAD). Our follow up study on *NGB* included 351 cases from the NIMH and the Indiana repositories as well as 289 healthy controls (Table 1) aged 48 to 99 (median =74, mean=73.2), 197 from NCRAD and 92 cognitively healthy spouses of the offspring of the NIMH subjects.

The samples used for sequencing *NGB* were 24 cases from the NIMH families showing the strongest linkage on 14q and 24 healthy controls. Samples used for gene expression analyses were punches from the temporal lobe of 30 deceased patients with confirmed AD pathology and 26 controls with no brain pathology. The time between death and harvest of the brain (Post Mortem Delay; PMD) varied from 2 to 24 hours. Cases were older than controls (83.3 ± 4.6 vs. 75.1 ± 14.3 years mean $\pm$ SD) and included more females (22 of 30 vs. 13 of 26). Both these variables were found to correlate significantly with the gene expression and were corrected for

in our model. PMD was higher in the controls (11.5 ± 5.1 vs. 7.7 ± 4.1 hours mean $\pm$ SD) but was not found to correlate with gene expression measurements ($p=0.8$).

All procedures involving human subjects were in accordance with the Declaration of Helsinki and were approved by the Johns Hopkins Institutional review board.

2.2 Candidate Gene Identification and SNP selection

The 1 LOD interval identified in our previous linkage study spanned a 26 Mb region containing approximately 150 known genes. Through a systematic literature search on each of the genes for co-occurrence in publications with keywords relevant to AD or psychosis (dementia, Alzheimer's, psychosis, hallucinations, schizophrenia, brain, neuron, hippocampus, presenilin, amyloid, amyloid beta, secretase, apoptosis, inflammation, oxidative stress, aging, cholesterol, mitochondria) we chose five genes for follow up: Dihydrolipoamide S-succinyltransferase (*DLST*), the hypoxia-inducible factor 1, alpha subunit (*HIF1A*), neuroglobin (*NGB*), numb homolog (*NUMB*), and sphingosine-1-phosphatase (*SGPPI*).

We downloaded reference genotype data from the HapMap project (Frazer, et al., 2007, The International HapMap Consortium, 2003) genome browser (www.hapmap.org, October 2005 release) in each of the candidate genes and surrounding regions to the extent of linkage disequilibrium (LD) islands according to the Gabriel et al definition (Gabriel, et al., 2002). We then analyzed the SNPs with allele frequency greater than 2% for pairwise LD and removed SNPs that had a partner with r^2 greater than 0.8. We genotyped the remaining 26 tagging SNPs (Table 2). In our follow up genotyping of 37 SNPs tagging the *NGB* gene (Table 3) we used the HapMap June 2006 release and extended the region 50 kb 5' and 3' of the gene to include potential regulatory sequences. The SNPs used in the follow up are listed in Table 3. The LD structure of the region made it necessary to extend into neighboring genes as noted in Table 3, however each of the SNPs outside *NGB* showed strong LD with the *NGB* region and their genotypes are likely to mirror genotypes of other SNPs within the gene.

2.3 Genotyping

Genotyping was performed in a 384 well format using the Taqman® method and “assays by design” from Applied Biosystems (Foster City, CA). Fluorescence end reads were performed on a ABI 7900HT sequence detection system and genotypes were called using SDS 3.1 software (Applied Biosystems). Follow up genotyping of 37 SNPs in *NGB* was performed as part of a larger genotyping project at the BROAD institute (www.broad.mit.edu/gen_analysis/genotyping) using the Illumina golden gate assay (Fan, et al., 2006). Illumina array calls were analyzed using Bead Studio software.

2.4 Sequencing

We analyzed by nucleotide sequencing all four known coding exons of *NGB* on 24 affected individuals from the families showing the strongest linkage in the region and 24 healthy controls. PCR reactions were used to amplify each exon and 30–60bp of its flanking sequence. Excess primers and nucleotides were inactivated by incubation with 1 U of shrimp alkaline phosphatase and 0.5 U of Exonuclease 1. The purified products were sequenced using the BigDye terminator 3.1 sequencing kit (Applied Biosystems) and the sequencing products were cleared of excess fluorescent nucleotides by isopropanol precipitation. The products were resuspended in EDTA and loaded on an ABI 3730 genetic analyzer. The sequences were aligned to the RefSeq (Pruitt, et al., 2007) transcript using the CodonCode sequence analysis software (CodonCode Corporation, Dedham, MA).

2.5 Gene Expression

To further explore the potential role of *NGB* in AD we measured the expression of *NGB* in 30 pathologically confirmed AD cases and 26 controls without brain pathology. RNA was extracted from the superior temporal lobe of punches from flash frozen brains using TRIzol reagent (Invitrogen) and first strand complementary DNA was generated using the TaqMan reverse transcription kit by Applied Biosystems (cat#N8080234). Primers were designed to quantitatively amplify from the *NGB* transcript cDNA a 100bp product crossing an exon – exon junction, thus ensuring that potential contaminating genomic DNA is not amplified. The PCR product from the transcript was quantified using SYBR-green real time detection with Applied Biosystems reagents (Foster City, CA, cat#4312704) and an ABI 7900 sequence detection system (Applied Biosystems). The *ACTB* housekeeping gene transcript was measured in the same manner to control for total RNA levels. Normalized *NGB* levels (ratios of *NGB/ACT*) were log transformed to achieve a normal distribution for further analysis, as described below.

2.6 Analytical Methods

Case control tests for genetic association were performed using the analytical tool kit UNPHASED (Dudbridge, 2008) (www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/). Tests of Hardy-Weinberg equilibrium (HWE) were performed using Haploview (Barrett, et al., 2005) (www.broad.mit.edu/mpg/haploview) which was also used for calculations of LD. Gene expression data was analyzed using generalized linear/non linear models in the STATISTICA version 7.1 software package (StatSoft Inc.; www.statsoft.com).

3. Results

Based on our literature search five candidate genes were chosen as functional and positional candidates and tested for association with AD. *DLST* (Dihydrolipoamide S-succinyltransferase) encodes a subunit of the alpha-ketoglutarate dehydrogenase complex, a mitochondrial respiratory component known to be defective in AD patients. It was chosen on the basis of several reports of associations between polymorphisms in *DLST* with AD (Ma, et al., 2001, Nakano, et al., 1997, Sheu, et al., 1999), although many others fail to identify an association (Brown, et al., 2004, Kunugi, et al., 1998, Matsushita, et al., 2001, Prince, et al., 2001). *HIF1A* (Hypoxia-inducible factor 1, alpha subunit) was chosen because it is one of the two subunits that comprise a heterodimeric transcription factor induced by hypoxia. It plays a role in neuroprotection by triggering genes involved in erythropoiesis, angiogenesis, glucose transport, and glycolysis (Soucek, et al., 2003). Similarly *NGB* (Neuroglobin) was chosen as an oxygen binding protein expressed mainly in the nervous system (Burmester, et al., 2000) and playing a protective role against hypoxia (Sun, et al., 2001, Sun, et al., 2003). *NUMB* (Numb homolog) was chosen because it functions in neural cell proliferation; (Li, et al., 2003, Petersen, et al., 2002) and *SGPPI* (Sphingosine-1-phosphatase) because its functions mediate cell growth and apoptosis (Cuvillier, 2002).

We successfully genotyped all SNPs but one (rs2883990), which was dropped from the analysis because it had a very low call rate (~50%). For the successful SNPs we achieved an average call rate of 98.4%, ranging from 93.6% (rs177378) to 100% (rs17750684). All SNPs were found to be in HWE in controls except for rs798847 in the *HIF1A* gene, which was only nominally ($p=0.049$) deviant from HWE and was kept for analysis. Analysis of the genotype data of each of the two case groups (psychotic, non-psychotic) against the controls for association failed to detect significant allelic associations, except for SNPs in *NGB* where many associations were detected in both comparisons as shown in Table 2. Among psychotic patients rs972725 and rs2216089 each showed nominally significant associations and are not strongly

correlated with each other ($r^2 = 0.3$). Among non-psychotic patients rs10133981 showed a nominal association ($p=0.0141$) while rs972725 again showed an association trend ($p=0.0615$).

We decided to further investigate *NGB* by genotyping additional markers to cover an extended region and to include new markers from the latest HapMap release. We included 37 SNPs in and around *NGB* in a larger study of 349 AD cases and 289 controls; this study did not target psychotic symptoms, but we decided that the prior association in both the psychotic and the non-psychotic cases justified the analysis regardless of psychotic status. There were 29 patients known to have psychotic symptoms and 53 AD patients known to be free of psychotic symptoms. There was a partial sample overlap with the previous analysis (82 cases, 136 controls). When comparing all AD cases with all controls, many SNPs showed a significant association, including rs733416 ($p=0.0012$) and rs888059 ($p=0.009$), which showed the highest significance (Table 3). These results did not change ($p=0.0013$ and $p=0.006$) when excluding 41 younger controls between the age of 48 and 60. For SNP rs733416 the risk allele was the rare allele (16% in controls – 23% in cases) with a relative risk of 1.6. The SNPs rs733416 and rs888059 are not correlated with rs972725 ($r^2=0.01$ and $r^2=0.06$) which had previously shown association in both psychotic and non-psychotic patients and are somewhat correlated with each other ($r^2=0.47$). In order to assess the study-wide significance of our result we determined the number of independent comparisons performed. First we calculated the r^2 between all pairs of SNPs. When two SNPs have for example an r^2 of 0.7, 70% of the variance of one SNP is accounted for by the other and they represent 1.3 independent tests. For each SNP we determined its highest r^2 from any pairwise SNP comparison, removed it from the set, and added $(1 - r^2)$ to the number of comparisons continuing until all SNPs were removed. We thus found that we had performed 36.4 independent comparisons, setting the desired p-value for study-wide significance to $p=0.00137$, which was exceeded by rs733416. Removing the 29 cases with psychosis increased the effect size for the rare allele of rs733416 to a relative risk of 1.64 and the significance improved to $p=7 \times 10^{-4}$.

Published data strongly support a neuroprotective role for *NGB*. This along with our association results prompted us to test whether reduced expression of the gene might increase the risk for AD, and whether the AD risk-associated variant is also associated with reduced *NGB* expression. Table 4 summarizes our expression results while raw data are provided on Supplementary Table 1. In a control-only analysis we found that *NGB* expression was strongly negatively correlated with age (2% reduction every year; $p=0.005$) and lower by 24% in females ($p<0.004$), while PMD did not have any effect. When modeling *NGB* expression as a function of age, sex and disease status we found that it increased by 23% ($p=0.007$) in AD patients compared to controls after correction for age and sex, suggesting up-regulation by the disease process. Age and sex effects remained highly significant (2.2%/year $p=0.001$ and 21% less in females $p=0.0008$). Inclusion of rs733416 genotype showed that *NGB* transcript expression was 13% lower in the carriers of the risk allele and reduced both in cases and in controls, however this difference did not reach statistical significance.

Sequencing of the four known exons of the *NGB* gene in 24 AD cases selected from families that showed the strongest evidence of linkage to 14q in our previous study and 24 older cognitively healthy controls revealed no exonic variants in either group.

4. Discussion

During our screening and follow up of positional and functional candidate genes for AD susceptibility we found a study-wide significant association between AD risk and a variant in the *NGB* gene. Gene expression analysis provided further evidence. Groups at higher risk like older subjects, females (Devi, et al., 2000, Gao, et al., 1998, Miech, et al., 2002, Seshadri, et al., 1997) and carriers of the risk allele, showed lower *NGB* expression. The presence of AD

pathology led to an increase of *NGB* transcript consistent with its known neuroprotective response to hypoxia (Sun, et al., 2001). Our data is therefore consistent with a model where women, older individuals and carriers of specific genotypes have lower levels of *NGB* leading to an increased risk for AD. It is likely that *NGB* up-regulation by the disease process in individuals with those risk factors is sometimes not sufficient to prevent neurodegeneration.

Neuroglobin is a small globin first identified by Burmester et al (Burmester, et al., 2000) as a member of the globin family after hemoglobin and myoglobin (and later cytoglobin), a member primarily expressed in the brain. It is a highly conserved protein, with only 6% of amino acids variant between mouse and humans (Burmester, et al., 2000) and, as we showed by sequencing, has no common coding variants in humans. It is a cytosolic protein localized near the mitochondria (Schmidt et al 2003), induced by neural hypoxia and cerebral ischemia and protecting neurons from hypoxic and ischemic injury (Greenberg, et al., 2008, Khan, et al., 2006, Sun, et al., 2001, Sun, et al., 2003). Despite its affinity to O₂ *NGB* is unlikely to function as a delivery system as it does not have sufficient concentration to account for changes in intracellular O₂ levels (Brunori and Vallone, 2007) and it does not increase rate of oxygen consumption (Sun et al 2001). Instead, it may be involved in scavenging reactive oxygen and nitrogen species (NO and peroxynitrite) generated in response to brain hypoxia (Herold, et al., 2004). Extensive research on neuroglobin by Khan et al (Greenberg, et al., 2008, Jin, et al., 2008, Khan, et al., 2007a, Khan, et al., 2008, Khan, et al., 2007b, Khan, et al., 2006) shows that neuroglobin's neuroprotection likely takes place at transduction of the death signal (Khan, et al., 2008). Most relevant to AD, neuroglobin attenuates amyloid beta neurotoxicity in vitro and the AD phenotype of transgenic mice (Khan, et al., 2007a), consistent with previous reports that it protects PC12 cells against amyloid beta induced cell injury (R.C. Li, et al., 2007). In agreement with our results, *NGB* levels have been shown to decline with in multiple rat brain regions which has been proposed to increase susceptibility to age related neurodegenerative disorders (Sun, et al., 2005). Our study is the first to examine genetic variation around *NGB* as a risk factor for AD in a human population. Our results are consistent with the literature, strongly supporting a neuroprotective role for neuroglobin through genetic association and gene expression.

According to the genome database's (genome.ucsc.edu) microRNA target prediction track (Krek, et al., 2005), *NGB* contains multiple predicted binding sites for hs-miR-214 in its 3' UTR. This is of particular interest in view of our expression results, as microRNAs have a direct effect on both translation and transcript degradation. hs-miR-214 is located within and presumably transcribed with *DNM3*, a gene highly expressed in the brain. Although we did not find any DNA variation at the microRNA recognition sites and despite the fact that according to the target prediction data of the microrna.org bioinformatics resource hs-miR-214 has more than 4,000 predicted targets in the genome, it would be of interest to determine the expression profile of hs-miR-214 in the healthy and AD affected brain.

NGB is very close in proximity to its neighboring genes, within an area of strong LD thus we cannot exclude that the genetic association we observe reflects their involvement. In fact, our strongest associated SNP is located within a neighboring gene *TMEM63C*, a transmembrane protein of unknown function. Although we feel it is important to acknowledge this gene, it is also important to note that we have no reason to believe that rs733416 is the actual functional DNA variant increasing risk for AD. According to the LD structure that variant is equally likely to be located within *NGB*. In fact, Hapmap lists 3 SNPs within and in the 3' flanking region of *NGB* that are in $r^2 \geq 0.7$ with rs733416, and others are likely to exist.

In conclusion our results, especially when combined with the literature, very strongly suggest that *NGB* warrants further attention for its likely involvement in neurological disorders. Further

work on this gene and its product will provide important insight and possibly intervention targets for a multitude of very common conditions, including stroke and AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Avramopoulos D, Fallin MD, Bassett SS. Linkage to chromosome 14q in Alzheimer's disease (AD) patients without psychotic symptoms. *Am J Med Genet B Neuropsychiatr Genet* 2005;132(1):9–13. [PubMed: 15389761]
- Bamberger ME, Landreth GE. Inflammation, apoptosis, and Alzheimer's disease. *Neuroscientist* 2002;8(3):276–83. [PubMed: 12061507]
- Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21(2):263–5. [PubMed: 15297300]
- Bertram L, Tanzi RE. The current status of Alzheimer's disease genetics: what do we tell the patients? *Pharmacol Res* 2004;50(4):385–96. [PubMed: 15304236]
- Blacker D, Bertram L, Saunders AJ, Moscarillo TJ, Albert MS, Wiener H, Perry RT, Collins JS, Harrell LE, Go RC, Mahoney A, Beaty T, Fallin MD, Avramopoulos D, Chase GA, Folstein MF, McInnis MG, Bassett SS, Doheny KJ, Pugh EW, Tanzi RE. Results of a high-resolution genome screen of 437 Alzheimer's disease families. *Hum Mol Genet* 2003;12(1):23–32. [PubMed: 12490529]
- Brown AM, Gordon D, Lee H, Caudy M, Haroutunian V, Blass JP. Substantial linkage disequilibrium across the dihydrolipoyl succinyltransferase gene region without Alzheimer's disease association. *Neurochem Res* 2004;29(3):629–35. [PubMed: 15038610]
- Brunori M, Vallone B. Neuroglobin, seven years after. *Cell Mol Life Sci* 2007;64(10):1259–68. [PubMed: 17385072]
- Burmester T, Weich B, Reinhardt S, Hankeln T. A vertebrate globin expressed in the brain. *Nature* 2000;407(6803):520–3. [PubMed: 11029004]
- Cecchi C, Fiorillo C, Sorbi S, Latorraca S, Nacmias B, Bagnoli S, Nassi P, Liguri G. Oxidative stress and reduced antioxidant defenses in peripheral cells from familial Alzheimer's patients. *Free Radic Biol Med* 2002;33(10):1372–9. [PubMed: 12419469]
- Cuvillier O. Sphingosine in apoptosis signaling. *Biochim Biophys Acta* 2002;1585(2–3):153–62. [PubMed: 12531549]
- Daw EW, Payami H, Nemens EJ, Nochlin D, Bird TD, Schellenberg GD, Wijsman EM. The number of trait loci in late-onset Alzheimer disease. *Am J Hum Genet* 2000;66(1):196–204. [PubMed: 10631151]
- Devi G, Ottman R, Tang MX, Marder K, Stern Y, Mayeux R. Familial aggregation of Alzheimer disease among whites, African Americans, and Caribbean Hispanics in northern Manhattan. *Arch Neurol* 2000;57(1):72–7. [PubMed: 10634451]
- Dudbridge F. Likelihood-based association analysis for nuclear families and unrelated subjects with missing genotype data. *Hum Hered* 2008;66(2):87–98. [PubMed: 18382088]
- Fan JB, Chee MS, Gunderson KL. Highly parallel genomic assays. *Nat Rev Genet* 2006;7(8):632–44. [PubMed: 16847463]

- Frazer KA, Ballinger DG, Cox DR, Hinds DA, Stuve LL, Gibbs RA, Belmont JW, Boudreau A, Hardenbol P, Leal SM, Pasternak S, Wheeler DA, Willis TD, Yu F, Yang H, Zeng C, Gao Y, Hu H, Hu W, Li C, Lin W, Liu S, Pan H, Tang X, Wang J, Wang W, Yu J, Zhang B, Zhang Q, Zhao H, Zhao H, Zhou J, Gabriel SB, Barry R, Blumenstiel B, Camargo A, Defelice M, Faggart M, Goyette M, Gupta S, Moore J, Nguyen H, Onofrio RC, Parkin M, Roy J, Stahl E, Winchester E, Ziaugra L, Altshuler D, Shen Y, Yao Z, Huang W, Chu X, He Y, Jin L, Liu Y, Shen Y, Sun W, Wang H, Wang Y, Wang Y, Xiong X, Xu L, Waye MM, Tsui SK, Xue H, Wong JT, Galver LM, Fan JB, Gunderson K, Murray SS, Oliphant AR, Chee MS, Montpetit A, Chagnon F, Ferretti V, Leboeuf M, Olivier JF, Phillips MS, Roumy S, Sallee C, Verner A, Hudson TJ, Kwok PY, Cai D, Koboldt DC, Miller RD, Pawlikowska L, Taillon-Miller P, Xiao M, Tsui LC, Mak W, Song YQ, Tam PK, Nakamura Y, Kawaguchi T, Kitamoto T, Morizono T, Nagashima A, Ohnishi Y, Sekine A, Tanaka T, Tsunoda T, Deloukas P, Bird CP, Delgado M, Dermitzakis ET, Gwilliam R, Hunt S, Morrison J, Powell D, Stranger BE, Whittaker P, Bentley DR, Daly MJ, de Bakker PI, Barrett J, Chretien YR, Maller J, McCarroll S, Patterson N, Pe'er I, Price A, Purcell S, Richter DJ, Sabeti P, Saxena R, Schaffner SF, Sham PC, Varilly P, Altshuler D, Stein LD, Krishnan L, Smith AV, Tello-Ruiz MK, Thorisson GA, Chakravarti A, Chen PE, Cutler DJ, Kashuk CS, Lin S, Abecasis GR, Guan W, Li Y, Munro HM, Qin ZS, Thomas DJ, McVean G, Auton A, Bottolo L, Cardin N, Eyheramendy S, Freeman C, Marchini J, Myers S, Spencer C, Stephens M, Donnelly P, Cardon LR, Clarke G, Evans DM, Morris AP, Weir BS, Tsunoda T, Mullikin JC, Sherry ST, Feolo M, Skol A, Zhang H, Zeng C, Zhao H, Matsuda I, Fukushima Y, Macer DR, Suda E, Rotimi CN, Adebamowo CA, Ajayi I, Aniagwu T, Marshall PA, Nkwodimma C, Royal CD, Leppert MF, Dixon M, Peiffer A, Qiu R, Kent A, Kato K, Niikawa N, Adewole IF, Knoppers BM, Foster MW, Clayton EW, Watkin J, Gibbs RA, Belmont JW, Muzny D, Nazareth L, Sodergren E, Weinstock GM, Wheeler DA, Yakub I, Gabriel SB, Onofrio RC, Richter DJ, Ziaugra L, Birren BW, Daly MJ, Altshuler D, Wilson RK, Fulton LL, Rogers J, Burton J, Carter NP, Clee CM, Griffiths M, Jones MC, McLay K, Plumb RW, Ross MT, Sims SK, Willey DL, Chen Z, Han H, Kang L, Godbout M, Wallenburg JC, L'Archeveque P, Bellemare G, Saeki K, Wang H, An D, Fu H, Li Q, Wang Z, Wang R, Holden AL, Brooks LD, McEwen JE, Guyer MS, Wang VO, Peterson JL, Shi M, Spiegel J, Sung LM, Zacharia LF, Collins FS, Kennedy K, Jamieson R, Stewart J. A second generation human haplotype map of over 3.1 million SNPs. *Nature* 2007;449(7164):851–61. [PubMed: 17943122]
- Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D. The structure of haplotype blocks in the human genome. *Science* 2002;296(5576):2225–9. [PubMed: 12029063]
- Gao S, Hendrie HC, Hall KS, Hui S. The relationships between age, sex, and the incidence of dementia and Alzheimer disease: a meta-analysis. *Arch Gen Psychiatry* 1998;55(9):809–15. [PubMed: 9736007]
- Gibson GE, Huang HM. Oxidative stress in Alzheimer's disease. *Neurobiol Aging* 2005;26(5):575–8. [PubMed: 15708429]
- Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 1991;349(6311):704–6. [PubMed: 1671712]
- Greenberg DA, Jin K, Khan AA. Neuroglobin: an endogenous neuroprotectant. *Curr Opin Pharmacol* 2008;8(1):20–4. [PubMed: 17942367]
- Herold S, Fago A, Weber RE, Dewilde S, Moens L. Reactivity studies of the Fe(III) and Fe(II)NO forms of human neuroglobin reveal a potential role against oxidative stress. *J Biol Chem* 2004;279(22):22841–7. [PubMed: 15020597]
- Jarvik G, Larson EB, Goddard K, Schellenberg GD, Wijsman EM. Influence of apolipoprotein E genotype on the transmission of Alzheimer disease in a community-based sample. *Am J Hum Genet* 1996;58(1):191–200. [PubMed: 8554056]
- Jin K, Mao XO, Xie L, Khan AA, Greenberg DA. Neuroglobin protects against nitric oxide toxicity. *Neurosci Lett* 2008;430(2):135–7. [PubMed: 18035490]
- Kehoe P, Wavrant-De Vrieze F, Crook R, Wu WS, Holmans P, Fenton I, Spurlock G, Norton N, Williams H, Williams N, Lovestone S, Perez-Tur J, Hutton M, Chartier-Harlin MC, Shears S, Roehl K, Booth J, Van Voorst W, Ramic D, Williams J, Goate A, Hardy J, Owen MJ. A full genome scan for late onset Alzheimer's disease. *Hum Mol Genet* 1999;8(2):237–45. [PubMed: 9931331]

- Khan AA, Mao XO, Banwait S, Jin K, Greenberg DA. Neuroglobin attenuates beta-amyloid neurotoxicity in vitro and transgenic Alzheimer phenotype in vivo. *Proc Natl Acad Sci U S A* 2007a;104(48):19114–9. [PubMed: 18025470]
- Khan AA, Ou Mao X, Banwait S, Dermardirossian CM, Bokoch GM, Jin K, Greenberg DA. Regulation of hypoxic neuronal death signaling by neuroglobin. *Faseb J*. 2008
- Khan AA, Sun Y, Jin K, Mao XO, Chen S, Ellerby LM, Greenberg DA. A neuroglobin-overexpressing transgenic mouse. *Gene* 2007b;398(1–2):172–6. [PubMed: 17537594]
- Khan AA, Wang Y, Sun Y, Mao XO, Xie L, Miles E, Graboski J, Chen S, Ellerby LM, Jin K, Greenberg DA. Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. *Proc Natl Acad Sci U S A* 2006;103(47):17944–8. [PubMed: 17098866]
- Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N. Combinatorial microRNA target predictions. *Nat Genet* 2005;37(5):495–500. [PubMed: 15806104]
- Kunugi H, Nanko S, Ueki A, Isse K, Hirasawa H. DLST gene and Alzheimer's disease. *Lancet* 1998;351(9115):1584–5. [PubMed: 10326565]
- Levy-Lahad E, Wijsman EM, Nemens E, Anderson L, Goddard KA, Weber JL, Bird TD, Schellenberg GD. A familial Alzheimer's disease locus on chromosome 1. *Science* 1995;269(5226):970–3. [PubMed: 7638621]
- Li HS, Wang D, Shen Q, Schonemann MD, Gorski JA, Jones KR, Temple S, Jan LY, Jan YN. Inactivation of Numb and Numlike in embryonic dorsal forebrain impairs neurogenesis and disrupts cortical morphogenesis. *Neuron* 2003;40(6):1105–18. [PubMed: 14687546]
- Li L, Zhang X, Yang D, Luo G, Chen S, Le W. Hypoxia increases A β generation by altering beta- and gamma-cleavage of APP. *Neurobiol Aging*. 2007
- Li RC, Pouranfar F, Lee SK, Morris MW, Wang Y, Gozal D. Neuroglobin protects PC12 cells against beta-amyloid-induced cell injury. *Neurobiol Aging*. 2007
- Ma Q, Chan P, Yang J. [Association between DLST gene polymorphism and Alzheimer's disease]. *Zhonghua Yi Xue Za Zhi* 2001;81(20):1246–8. [PubMed: 11825528]
- Matsushita S, Arai H, Yuzuriha T, Kato M, Matsui T, Urakami K, Higuchi S. No association between DLST gene and Alzheimer's disease or Wernicke-Korsakoff syndrome. *Neurobiol Aging* 2001;22(4):569–74. [PubMed: 11445257]
- McGeer PL, Rogers J, McGeer EG. Inflammation, anti-inflammatory agents and Alzheimer disease: the last 12 years. *J Alzheimers Dis* 2006;9(3 Suppl):271–6. [PubMed: 16914866]
- Miech RA, Breitner JC, Zandi PP, Khachaturian AS, Anthony JC, Mayer L. Incidence of AD may decline in the early 90s for men, later for women: The Cache County study. *Neurology* 2002;58(2):209–18. [PubMed: 11805246]
- Nakano K, Ohta S, Nishimaki K, Miki T, Matuda S. Alzheimer's disease and DLST genotype. *Lancet* 1997;350(9088):1367–8. [PubMed: 9365459]
- Numomura A, Castellani RJ, Zhu X, Moreira PI, Perry G, Smith MA. Involvement of oxidative stress in Alzheimer disease. *J Neuropathol Exp Neurol* 2006;65(7):631–41. [PubMed: 16825950]
- Peers C, Pearson HA, Boyle JP. Hypoxia and Alzheimer's disease. *Essays Biochem* 2007;43:153–64. [PubMed: 17705799]
- Pericak-Vance MA, Grubber J, Bailey LR, Hedges D, West S, Santoro L, Kemmerer B, Hall JL, Saunders AM, Roses AD, Small GW, Scott WK, Conneally PM, Vance JM, Haines JL. Identification of novel genes in late-onset Alzheimer's disease. *Exp Gerontol* 2000;35(9–10):1343–52. [PubMed: 11113612]
- Petersen PH, Zou K, Hwang JK, Jan YN, Zhong W. Progenitor cell maintenance requires numb and numlike during mouse neurogenesis. *Nature* 2002;419(6910):929–34. [PubMed: 12410312]
- Prince JA, Feuk L, Sawyer SL, Gottfries J, Ricksten A, Nagga K, Bogdanovic N, Blennow K, Brookes AJ. Lack of replication of association findings in complex disease: an analysis of 15 polymorphisms in prior candidate genes for sporadic Alzheimer's disease. *Eur J Hum Genet* 2001;9(6):437–44. [PubMed: 11436125]
- Pruitt KD, Tatusova T, Maglott DR. NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* 2007;35(Database issue):D61–5. [PubMed: 17130148]

- Reynolds A, Laurie C, Mosley RL, Gendelman HE. Oxidative stress and the pathogenesis of neurodegenerative disorders. *Int Rev Neurobiol* 2007;82:297–325. [PubMed: 17678968]
- Seshadri S, Wolf PA, Beiser A, Au R, McNulty K, White R, D'Agostino RB. Lifetime risk of dementia and Alzheimer's disease. The impact of mortality on risk estimates in the Framingham Study. *Neurology* 1997;49(6):1498–504. [PubMed: 9409336]
- Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, et al. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 1995;375(6534):754–60. [PubMed: 7596406]
- Sheu KF, Brown AM, Kristal BS, Kalaria RN, Lilius L, Lannfelt L, Blass JP. A DLST genotype associated with reduced risk for Alzheimer's disease. *Neurology* 1999;52(7):1505–7. [PubMed: 10227647]
- Shi Q, Gibson GE. Oxidative stress and transcriptional regulation in Alzheimer disease. *Alzheimer Dis Assoc Disord* 2007;21(4):276–91. [PubMed: 18090434]
- Shimohama S. Apoptosis in Alzheimer's disease--an update. *Apoptosis* 2000;5(1):9–16. [PubMed: 11227497]
- Soucek T, Cumming R, Dargusch R, Maher P, Schubert D. The regulation of glucose metabolism by HIF-1 mediates a neuroprotective response to amyloid beta peptide. *Neuron* 2003;39(1):43–56. [PubMed: 12848931]
- Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci U S A* 1993;90(5):1977–81. [PubMed: 8446617]
- Sun Y, Jin K, Mao XO, Xie L, Peel A, Childs JT, Logvinova A, Wang X, Greenberg DA. Effect of aging on neuroglobin expression in rodent brain. *Neurobiol Aging* 2005;26(2):275–8. [PubMed: 15582755]
- Sun Y, Jin K, Mao XO, Zhu Y, Greenberg DA. Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. *Proc Natl Acad Sci U S A* 2001;98(26):15306–11. [PubMed: 11742077]
- Sun Y, Jin K, Peel A, Mao XO, Xie L, Greenberg DA. Neuroglobin protects the brain from experimental stroke in vivo. *Proc Natl Acad Sci U S A* 2003;100(6):3497–500. [PubMed: 12621155]
- Takuma K, Yan SS, Stern DM, Yamada K. Mitochondrial dysfunction, endoplasmic reticulum stress, and apoptosis in Alzheimer's disease. *J Pharmacol Sci* 2005;97(3):312–6. [PubMed: 15750290]
- The_International_HapMap_Consortium. The International HapMap Project. *Nature* 2003;426(6968):789–96. [PubMed: 14685227]
- Waring SC, Rosenberg RN. Genome-wide association studies in Alzheimer disease. *Arch Neurol* 2008;65(3):329–34. [PubMed: 18332245]
- Weisman D, Hakimian E, Ho GJ. Interleukins, inflammation, and mechanisms of Alzheimer's disease. *Vitam Horm* 2006;74:505–30. [PubMed: 17027528]
- Wyss-Coray T. Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nat Med* 2006;12(9):1005–15. [PubMed: 16960575]
- Zhu X, Smith MA, Honda K, Aliev G, Moreira PI, Nunomura A, Casadesus G, Harris PL, Siedlak SL, Perry G. Vascular oxidative stress in Alzheimer disease. *J Neurol Sci* 2007;257(1–2):240–6. [PubMed: 17337008]

TABLE 1

Description of association studies
Numbers of SNPs and samples used in the two steps of our association study.

	number of SNPs	cases with psychosis	cases without psychosis	total cases	controls
original screen of 5 genes	26	99	125	224 *	152
follow-up of NGB	37	29 *	53 *	351 †	289

* = these were not directly compared to controls due to study design.
† Includes 269 cases with no information on the presence of psychotic symptoms.

TABLE 2

Association study across 5 candidate genes.

SNPs genotyped for the screening of the five selected candidate genes, their location in the genome and results of the association tests comparing controls with AD cases with Hallucinations (labeled psy for psychotic) or AD cases without Hallucinations (labeled non-psy).

SNP Name	Target Gene	LOCATION (Mb, Chr.14)	99 psy cases vs. 152 controls	125 non-psy cases vs. 152 controls
RS2256605	<i>HIF1A</i>	60,130,763	n.s.	n.s.
RS11846496	<i>HIF1A</i>	60,133,314	n.s.	*
RS798847	<i>HIF1A</i>	60,137,252	n.s.	n.s.
RS2301113	<i>HIF1A</i>	60,196,589	n.s.	n.s.
RS1319462	<i>HIF1A</i>	60,209,266	n.s.	*
RS17750684	<i>SGPP1</i>	62,086,503	n.s.	n.s.
RS2883990	<i>SGPP1</i>	62,147,158	ND	ND
RS11624105	<i>SGPP1</i>	62,158,906	*	n.s.
RS8013824	<i>SGPP1</i>	62,194,523	n.s.	n.s.
RS6574115	<i>NUMB</i>	71,714,136	n.s.	n.s.
RS2293797	<i>NUMB</i>	71,715,700	n.s.	n.s.
RS1047849	<i>NUMB</i>	71,731,227	n.s.	n.s.
RS177380	<i>NUMB</i>	71,736,353	*	n.s.
RS177378	<i>NUMB</i>	71,740,043	n.s.	n.s.
RS10141031	<i>NUMB</i>	71,763,650	n.s.	n.s.
RS2108552	<i>NUMB</i>	71,866,967	n.s.	n.s.
RS4899468	<i>NUMB</i>	71,926,638	n.s.	n.s.
RS2159905	<i>DLST</i>	73,333,068	n.s.	n.s.
RS732765	<i>DLST</i>	73,355,770	n.s.	n.s.
RS3213716	<i>DLST</i>	73,368,226	n.s.	n.s.
RS3213717	<i>DLST</i>	73,368,388	n.s.	n.s.
RS3813539	<i>NGB</i>	76,793,979	n.s.	n.s.
RS3783988	<i>NGB</i>	76,804,333	*	n.s.
RS10133981	<i>NGB</i>	76,805,546	n.s.	**
RS972725	<i>NGB</i>	76,818,064	**	*
RS2216089	<i>NGB</i>	76,819,810	**	n.s.

* nominally suggestive ($p < 0.1$).

** nominally significant ($p < 0.05$)

TABLE 3

Association results within the extent of LD around NGB.

SNP genotyped in and around the NGB gene for follow up and the results (p-values) of the test for association. Due to the long extent of LD and the tight positioning of the genes in the area most SNPs are within other genes but still in LD with variation in the NGB genomic region.

SNP	Position	gene	351 cases vs. 289 controls
rs8021076	76,789,099	<i>TMEM63C</i>	p-value n.s.
rs888059	76,791,901	<i>TMEM63C</i>	***
rs733416	76,792,220	<i>TMEM63C</i>	****
rs11847091	76,793,725	<i>TMEM63C</i>	n.s.
rs3813539	76,793,979	<i>TMEM63C</i>	n.s.
rs7141596	76,794,757	<i>TMEM63C</i>	**
rs369202	76,796,110		*
rs888060	76,796,486		n.s.
rs747273	76,796,731		n.s.
rs368855	76,797,599		**
rs3783988	76,804,333	<i>NGB</i>	**
rs10133981	76,805,546	<i>NGB</i>	n.s.
rs7159558	76,812,682	<i>POMT</i>	n.s.
rs438931	76,816,063	<i>POMT</i>	n.s.
rs4540995	76,816,565	<i>POMT</i>	n.s.
rs2098380	76,816,776	<i>POMT</i>	n.s.
rs2058916	76,824,283	<i>POMT</i>	n.s.
rs11627257	76,832,017	<i>POMT</i>	n.s.
rs3783986	76,832,625	<i>POMT</i>	n.s.
rs17105685	76,834,445	<i>POMT</i>	n.s.
rs8015231	76,839,659	<i>POMT</i>	n.s.
rs12433986	76,842,145	<i>POMT</i>	n.s.
rs4899650	76,846,254	<i>POMT</i>	n.s.
rs8177544	76,858,541	<i>GSTZ1</i>	n.s.
rs8016187	76,860,625	<i>GSTZ1</i>	n.s.
rs8004558	76,861,793	<i>GSTZ1</i>	n.s.
rs2270422	76,862,577	<i>GSTZ1</i>	n.s.
rs3177429	76,862,990	<i>GSTZ1</i>	n.s.
rs2287396	76,863,945	<i>GSTZ1</i>	n.s.
rs8177565	76,864,650	<i>GSTZ1</i>	n.s.
rs8177569	76,865,421	<i>GSTZ1</i>	n.s.
rs8177573	76,866,511	<i>GSTZ1</i>	n.s.
rs2287397	76,866,664	<i>GSTZ1</i>	n.s.
rs2287398	76,879,072	<i>TMED8</i>	n.s.
rs17105732	76,889,000	<i>TMED8</i>	n.s.

SNP	Position	gene	351 cases vs. 289 controls
rs11850308	76,890,139	<i>TMED8</i>	n.s.
rs1544708	76,902,835	<i>TMED8</i>	n.s.

n.s.= not significant

*
p<0.1,

**
p<0.05,

p<0.01,

p<0.002.

TABLE 4

Temporal lobe gene expression analyses.
The top panel represent a controls-only analysis showing that older age and female sex are associated with decrease NGB expression. The bottom panel shows the comparison of cases with controls correcting for age and sex..

Variable	Level of effect	Fold change	Standard Error	Wald Statistic	p
Controls only analysis (N=26)					
AGE	per year	0.98	0.010	7.97	****
SEX	female	0.76	0.136	8.08	****
PMD	per hour	0.99	0.027	0.08	n.s.
Cases (N=26) and controls (N=30) analysis					
AGE	per year	0.98	0.010	10.90	*****
SEX	female	0.79	0.103	11.13	*****
AD	case	1.23	0.110	7.23	****
AD * SEX			0.103	1.30	n.s.

n.s.= not significant
* p<0.1,
** p<0.05,
*** p<0.01,
**** p<0.002,
***** p<0.001