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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 five 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 *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 (Li et al., 2007a,b; 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

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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 five 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 58–99 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 48–99 (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 h. Cases were older than controls (83.3 ± 4.6 years vs. 75.1 ± 14.3 years mean $\pm$ S.D.) 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 h vs. 7.7 ± 4.1 h mean $\pm$ S.D.) 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 (*SGPP1*).

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

Table 1
Description of association studies.

	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

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

SNP name	Target gene	Location (Mb, Chr.14)	99 psy cases vs. 152 controls	125 non-psy cases vs. 152 controls
rs2256605	*HIF1A*	60,130,763	n.s.	n.s.
rs11846496	*HIF1A*	60,133,314	n.s.	*
rs798847	*HIF1A*	60,137,252	n.s.	n.s.
rs2301113	*HIF1A*	60,196,589	n.s.	n.s.
rs1319462	*HIF1A*	60,209,266	n.s.	*
rs17750684	*SGPP1*	62,086,503	n.s.	n.s.
rs2883990	*SGPP1*	62,147,158	ND	ND
rs11624105	*SGPP1*	62,158,906	*	n.s.
rs8013824	*SGPP1*	62,194,523	n.s.	n.s.
rs6574115	*NUMB*	71,714,136	n.s.	n.s.
rs2293797	*NUMB*	71,715,700	n.s.	n.s.
rs1047849	*NUMB*	71,731,227	n.s.	n.s.
rs177380	*NUMB*	71,736,353	*	n.s.
rs177378	*NUMB*	71,740,043	n.s.	n.s.
rs10141031	*NUMB*	71,763,650	n.s.	n.s.
rs2108552	*NUMB*	71,866,967	n.s.	n.s.
rs4899468	*NUMB*	71,926,638	n.s.	n.s.
rs2159905	*DLST*	73,333,068	n.s.	n.s.
rs732765	*DLST*	73,355,770	n.s.	n.s.
rs3213716	*DLST*	73,368,226	n.s.	n.s.
rs3213717	*DLST*	73,368,388	n.s.	n.s.
rs3813539	*NGB*	76,793,979	n.s.	n.s.
rs3783988	*NGB*	76,804,333	*	n.s.
rs10133981	*NGB*	76,805,546	n.s.	**
rs972725	*NGB*	76,818,064	**	*
rs2216089	*NGB*	76,819,810	**	n.s.

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).
* Nominally suggestive ($p < 0.1$).
** Nominally significant ($p < 0.05$).

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 30–60 bp 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)

Table 3
Association results within the extent of LD around NGB.

SNP	Position	Gene	351 cases vs. 289 controls *p*-value
rs8021076	76,789,099	*TMEM63C*	n.s.
rs888059	76,791,901	*TMEM63C*	***
rs733416	76,792,220	*TMEM63C*	****
rs11847091	76,793,725	*TMEM63C*	n.s.
rs3813539	76,793,979	*TMEM63C*	n.s.
rs7141596	76,794,757	*TMEM63C*	**
rs369202	76,796,110		*
rs888060	76,796,486		n.s.
rs747273	76,796,731		n.s.
rs368855	76,797,599		**
rs3783988	76,804,333	*NGB*	**
rs10133981	76,805,546	*NGB*	n.s.
rs7159558	76,812,682	*POMT*	n.s.
rs438931	76,816,063	*POMT*	n.s.
rs4540995	76,816,565	*POMT*	n.s.
rs2098380	76,816,776	*POMT*	n.s.
rs2058916	76,824,283	*POMT*	n.s.
rs11627257	76,832,017	*POMT*	n.s.
rs3783986	76,832,625	*POMT*	n.s.
rs17105685	76,834,445	*POMT*	n.s.
rs8015231	76,839,659	*POMT*	n.s.
rs12433986	76,842,145	*POMT*	n.s.
rs4899650	76,846,254	*POMT*	n.s.
rs8177544	76,858,541	*GSTZ1*	n.s.
rs8016187	76,860,625	*GSTZ1*	n.s.
rs8004558	76,861,793	*GSTZ1*	n.s.
rs2270422	76,862,577	*GSTZ1*	n.s.
rs3177429	76,862,990	*GSTZ1*	n.s.
rs2287396	76,863,945	*GSTZ1*	n.s.
rs8177565	76,864,650	*GSTZ1*	n.s.
rs8177569	76,865,421	*GSTZ1*	n.s.
rs8177573	76,866,511	*GSTZ1*	n.s.
rs2287397	76,866,664	*GSTZ1*	n.s.
rs2287398	76,879,072	*TMED8*	n.s.
rs17105732	76,889,000	*TMED8*	n.s.
rs11850308	76,890,139	*TMED8*	n.s.
rs1544708	76,902,835	*TMED8*	n.s.

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

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 100-bp 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. Dihydrolipoamide *S*-succinyltransferase (*DLST*) 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). Hypoxia-inducible factor 1, alpha subunit (*HIF1A*) 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 neuroglobin (*NGB*) 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 homolog (*NUMB*) was chosen because it functions in neural cell proliferation; (Li et al., 2003; Petersen et al., 2002) and sphingosine-1-phosphatase (*SGPP1*) 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 4
Temporal lobe gene expression analyses.

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.

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. n.s. = not significant.
**** $p < 0.002$.
***** $p < 0.001$.

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 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 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 in 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 it's 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. (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 O_2 *NGB* is unlikely to function as a delivery system as it does not have sufficient concentration to account for changes in intracellular O_2 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., 2006, 2007a,b, 2008) 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 (Li et al., 2007a,b). 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 4000 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.

5. Conflicts of interest

The authors have no actual or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2008.10.003.

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