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Potential involvement of GRIN2B encoding the NMDA receptor subunit NR2B in the spectrum of Alzheimer's disease

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Abstract Increasing evidence links dysregulation of NR2B-containing *N*-methyl-D-aspartate receptor remodelling and trafficking to Alzheimer's disease (AD). This theme offers the possibility that the GRIN2B gene, encoding this selective NR2B subunit, represents a potential molecular modulating factor for this disease. Based on this hypothesis, we carried out a mutation scanning of exons and flanking regions of GRIN2B in a well-characterized cohort of AD patients, recruited from Southern Italy. A "de novo" p.K1293R mutation, affecting a highly conserved residue of the protein in the C-terminal domain, was observed for the first time in a woman with familial AD, as the only genetic alteration of relevance. Moreover, an association study between the other detected sequence variants and AD was performed. In particular, the study was focused on five identified single nucleotide polymorphisms: rs7301328, rs1805482, rs3026160, rs1806191 and rs1806201, highlighting a significant contribution from the GRIN2B rs1806201 T allele towards disease susceptibility [adjusted odds ratio (OR) = 1.92, 95% confidence interval (CI) 1.40–2.63, $p < 0.001$, after correction for sex, age, and APOE ε4 genotype]. This was confirmed by haplotype analysis that identified a specific haplotype, carrying the rs1806201 T allele (CCCTC), over-represented in patients versus controls (adjusted OR = 6.03; $p < 0.0001$). Although the pathogenic role of the GRIN2B-K1293R mutation in AD is not clear, our data advocate that genetic variability in the GRIN2B gene, involved in synaptic functioning, might provide valuable insights into disease pathogenesis, continuing to attract significant attention in biomedical research on its genetic and functional role.

Keywords Alzheimer's dementia · Excitotoxicity · Glutamate · *N*-methyl-D-aspartate receptors *GRIN2B* gene · Regulation of synaptogenesis

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Introduction

Alzheimer's disease (AD), one of the most serious health problems in the industrialized world, is an insidious and progressive neurodegenerative disorder that accounts for the vast majority of age-related dementia. Genetically heterogeneous, displaying no single or simple mode of inheritance, AD is usually divided into familial and sporadic forms according to family history. Notably, the clinical presentation of familial forms of AD (FAD) is more complex, and mutations of the presenilin 1 (PSEN-1, at locus 14q24.3), presenilin 2 (PSEN-2, at locus 1q31-q42) and amyloid precursor protein (APP, at locus 21q21.2) have also been described in these patients (Cruts et al. 1998; Goate et al. 1991). On the basis of their function, these proteins regulate the production of the amyloid β- (Aβ) peptide by an elusive mechanism that modulates the proteolysis of APP, but how these elements orchestrate the overall activity is still a matter of investigation. Conversely, most sporadic AD (SAD) forms have a multifactorial aetiology, caused by environmental and genetic factors, which are not sufficient alone for

the development of the disease. In this contest, only the apolipoprotein E (APOE) ε4 allele is recognized as a major genetic risk factor increasing the susceptibility to AD (Sadigh-Eteghad et al. 2012). There is now an increasing requirement in the quest to uncover the elusive AD genes, their common biochemical pathways and the putative pathogenetic roles of some of these potential AD risk factors. Currently, the interest of research is also focused on finding symptomatic treatment aimed at modifying the course of the disease, to slow down its pathogenic process. In this regard, neurobiological and functional genomics studies have supported the introduction of pharmacogenomic approaches in AD drug development, which may help to optimize therapeutics (Cacabelos 2008; Darreh-Shori et al. 2012). In any case, even though multiple pathways and mechanisms of AD may lead to the initiation of synaptic damage and neuronal cell loss, it is certain that glutamate-mediated excitotoxicity is implicated in typical AD neuronal dysfunction and cognitive impairment (Olney et al. 1997; Hu et al. 2012). This type of excitotoxicity is caused, at least in part, by excessive activation of the *N*-methyl-D-aspartate receptors (NMDAR), critical members of the ionotropic glutamate receptor family for regulation of synaptogenesis, neuronal networks, learning and memory. Functionally, NMDAR are heteromeric complexes, usually comprised of two obligatory NR1 and two different NR2 (A, B, C, D) subunits. The NMDAR multigroups consisting of NR1 and NR2A/B subunits are the most abundant in the hippocampus and throughout the forebrain. These different forms exhibit distinct biophysical properties and receptor targets, which likely reflect their function in different areas of the brain (Monyer et al. 1992; Paoletti 2011). Furthermore, new research provides evidence that synaptic NMDAR (NMDARs) number and subunit composition are not static, but change dynamically in a synapse-specific manner during neurodevelopment and in response to neuronal activity or sensory experience (Cull-Candy and Leszkiewicz 2004; Lau and Zukin 2007; Petralia 2012). Notably, the identity of the NR2 subunit determines many of pharmacological properties of this receptor family and can also influence NMDARs assembly, downstream signalling, receptor trafficking and synaptic targeting through the unique coupling of proteins to the C-terminus of each NR2B subunit (Singh et al. 2012). Consistent with this involvement, NR2B-containing NMDARs, on which the glutamate-binding site is contained, offer a particularly rich pharmacology with distinct recognition sites for allosteric ligands, and help to govern the overall formation of the functional receptors (Laube et al. 1997; Mony et al. 2009). An interesting theme that has recently emerged identifies NR2B as a candidate and promising target for modern AD therapeutic strategies (Reisberg et al. 2003; Santangelo et al. 2012). Indeed, several emerging studies have proven the efficacy of antagonists selective for these receptors, which segregate to extrasynaptic compartments, exclusively composed of NR2B subunits, for cognitive-enhancing therapy in AD (Winblad et al. 2007; Porsteinsson et al. 2008). The prevailing view suggests that an increase in glutamate levels for chronic activation of extrasynaptic NMDAR may lead first to death of postsynaptic neurons, followed by synaptotoxicity and ultimately cell death, which correlates with the loss of memory function and learning ability in AD patients (Danysz and Parsons 2012). In the complex, these different mechanisms all seem to culminate in a specific gain of toxic function, which should cause a sustained neuronal Aβ release, one of the pathological keys of AD, identifying a possible agent in the modulation of Aβ metabolism in NR2B (Snyder et al. 2005; Tackenberg et al. 2013). The human gene encoding the NR2B subunit, named *GRIN2B*, at locus 12p12, is expressed nearly exclusively in the central nervous system (CNS), including regions predominantly affected in AD, such as the hippocampus pyramidal cells and, at a lower extent, the basal ganglia (amygdala and striatum). The non-ubiquitous anatomical distribution of the *GRIN2B* mRNA in CNS suggests that the gene could be involved in specific functions pertaining to the expressing cells group (Schito et al. 1997). According to this concept, there is also a great deal of evidence documenting that both the NR2B subunit and its mRNA level are significantly downregulated in these susceptible regions of the AD brain, showing that the gene expression, not just the protein, is also selectively altered in AD (Sze et al. 2001; Bi and Sze 2002; Stein et al. 2012). Thus, all the findings highlighted above raise the intriguing possibility that genetic variations in *GRIN2B* could influence the vulnerability to the disease. Previous genetic studies have been conducted to explain an active role of this gene into the molecular mechanism of AD (Seripa et al. 2008; Jiang and Jia 2009; Chen et al. 2010). The data currently available are, however, contradictory, and for now there is not enough information to state with any certainty that single mutations in this gene are involved in the overlapping processes related to AD pathogenesis. In light of this preliminary evidence, we aimed to obtain fascinating new data in the context of the genetic architecture of AD, by performing an extensive mutation analysis of the *GRIN2B* coding region in a well-characterized cohort of patients with AD and evaluating frequencies and distributions of the identified sequence variants in our Southern Italy population.

Materials and methods

Subject recruitment and clinical information

DNA samples from a total of 520 subjects, descendent from many generations from the Calabria region, Southern Italy, were analysed. Patients ($n = 270$; 61 % women,

Table 1 Clinical characteristics of AD patients

AD patients	Variable
No of subjects	270
Age (years)	67.52 ± 9.0
MMSE	14.05 ± 6.2
Women/men	162/108
Familial AD (%)	81 (30)
Sporadic AD (%)	189 (70)
EOAD *n* (%)	98 (36.3)
Age at onset (years)	54.35 ± 7.25
LOAD *n* (%)	172 (63.7)
Age at onset (years)	69.88 ± 3.40
APOE-ε4 carriers *n* (%)	113 (41.9)

Mean ± SD of age, age at onset, and MMSE

Age of onset of Alzheimer disease symptoms for affected individuals

MMSE score Mini-Mental State Examination adjusted for age and education, *EOAD* early-onset Alzheimer's disease, *LOAD* late-onset Alzheimer's disease

67.52 ± 9.0 years; mean ± SD) were selected from a group of 350 outpatients with dementia recruited from the Institute of Neurology, University "Magna Graecia" in Catanzaro, and subsequently screened for mutations at the Institute of Neurological Sciences, National Research Council, in Cosenza, Italy. Exclusion criterion was the evidence of primary neurologic diseases and mental disorders other than AD. Clinical AD diagnosis was made according to The National Institute of Neurological and Communicative Disorders and Stroke and The Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (McKhann et al. 1984). Clinical characteristics of AD patients are summarized in Table 1. Briefly, each patient underwent a diagnostic neuropsychological examination, including Mini-Mental State Examination (MMSE; score 14.05 ± 6.2; mean ± SD) (Crum et al. 1993). The disease was considered to be familial if at least one additional first degree relative suffered from AD-type dementia, otherwise it was defined as sporadic with negative family history. Information on the family history of AD was obtained by conducting a reliable, validated interview with each case/control and relative. For relatives who were deceased or otherwise unavailable for interview, the family history was obtained by interviewing the most knowledgeable informant. Onset age was established as the age at which significant memory, cognitive and/or behavioural changes began interfering with daily life. To compare the presence and/or allele frequencies of the genetic variants identified in this study, healthy control subjects ($n = 226$; 57 % women; age, 63.8 ± 8.2 years; mean ± SD) enrolled during a previous study on ageing (Andreoli et al. 2011) and neurologically scored as "no cognitive decline" (MMSE ≥28) were involved in our mutational screening. All participants were included in this study, which was approved by an institutional review board and conducted in accordance with the provisions of the Helsinki declaration, after obtaining their informed consent or the consent of their legal wardens.

Blood sample collection and genetic analysis

GRIN2B, located in reverse orientation on chromosome 12p13.1, is a moderately large gene comprising 13 exons; the coding sequence is encompassed by exons 2 through 13. Briefly, through PCR, denaturing high-performance liquid chromatography (DHPLC) and direct DNA sequencing, we performed a comprehensive coding region mutational analysis, using primers designed to completely incorporate the exons and the splice junctions of *GRIN2B* (Primer3 software; amplification, sequences and DHPLC analysis conditions are available on request). Blood samples for the genomic DNA studies were obtained from peripheral blood leukocytes and DNA was extracted according to standard procedure. First, the presence of new variants in DNA samples from AD patients was investigated. DNA was amplified using PCR in a total volume of 50 µl containing 15 pmol of each primer, 200 ng genomic DNA and AmpliTaq Gold (Applied Biosystems), using standard conditions on a PTC-100TM Programmable Thermal Controller (MJ Res. Inc., Genenco). Mutational screening, performed on all amplified fragments from each patient, was done by DHPLC (Frueh and Noyer-Weidner 2003) on a Wave® DNA Fragment Analysis System (Transgenomic Inc., San Jose, CA) with a DNASep HT cartridge (Transgenomic). The present approach allowed us to search for all nucleotide variations in the *GRIN2B*-coding regions. In particular, optimal conditions for each injection (temperature, elution time, buffer composition) were determined using the WAVE Maker software (version 4.1.40; Transgenomic). After amplification, each amplicon was analysed both individually and as part of the mixture composed by an equal volume of normal PCR product amplified from DNA coming from a healthy subject and previously sequenced, to verify the absence of mutations or polymorphisms, thereby allowing also the detection of both hetero- and homozygous mutations. Patients' chromatograms showing abnormal DHPLC elution profiles were analysed by double-strand DNA direct sequencing with Applied Biosystems BigDye terminator v1.1 sequencing chemistry, then run on an ABI3130xl (Applied Biosystems) genetic analyser as per manufacturer's instructions. All sequence variants identified were confirmed on a second amplified PCR sample. Furthermore, the screening for mutations in PSEN-1/PSEN-2 (exons 3–12) and APP (exons 16 and 17) genes was also carried out, either using

PCR-DHPLC method or sequencing analysis, as described above. In the next stage of this study, to shed light on a potential genetic role of identified variants, the same mutational analysis was extended to the control subjects. Then, to select SNPs that might be associated with the AD phenotype in our study, according to the conventional criterion for complex human diseases such as AD, we focused our analysis on the most common polymorphisms genotyped in the pooled sample of cases and controls, with population minor allele frequency (MAF) $\geq$5 % (Luo et al. 2012; Guerreiro et al. 2012). In addition, the SIFT (http://sift.bii.a-star.edu.sg/), PolyPhen (http://genetics.bwh.harvard.edu/pph2/) and SNAP (http://www.rostlab.org/services/SNAP) programmes were used to predict the functional impact of the missense variants on the structure and function of *GRIN2B*. In all instances, default parameters were used for each programme. Finally, APOE genotyping was carried out by *Cfo*I restriction enzyme digestion as previously described (Andreoli et al. 2011).

Statistical analyses

Demographic characteristics of patients and controls were compared using the Pearson χ^2 test for sex and the Mann–Whitney *U* test for age. Linkage disequilibrium (LD) between markers was computed using HAPLOVIEW v.4.2. Comparisons of allele and genotype frequencies for each marker in patients and controls were performed using the Pearson χ^2 test or the Fisher's exact test, when expected frequencies were very small. The Breslow–Day method was applied to test the homogeneity of the ORs between strata. Statistical power was estimated with Quanto v.1.2.4. The significance level was set at 0.05. After Bonferroni correction, a p value = 0.01 (0.05/numbers of SNPs tested) was considered statistically significant in marker analysis. Multiple-test correction for haplotypes was conducted by the permutation test (50,000 permutations). Values were adjusted for sex, age and APOE ε4 carrier status (only for sex and age in the APOE ε4 strata) using logistic regression. Allele, genotype and haplotype analyses were carried out using PLINK v.1.07.

Results

Mutation screening

A total of 11 molecular variants were found in the exon regions of the *GRIN2B* gene. First and foremost, a novel putative missense mutation in exon 13 (c.3878A>G) was identified in a patient with AD familial history (Supplementary Fig. 1). The proband was a 48-year-old woman who came under our observation with a diagnosis of probable AD. The initial symptoms of cognitive deficit (age at onset 47 years) subsequently are complicated by progressive memory impairment and behavioural disorders. One of the sisters of the proband died at age 57 following a 12-year history of progressive cognitive deterioration. Her pedigree presented a positive history of dementia in several members spanning through two generations (i.e. cognitive and behavioural symptoms were reported in the proband's mother, in two of her mother's siblings and in her sister, all deceased), with an inheritance pattern suggesting an autosomal dominant trait (other relatives of this patient were not available to participate in our molecular genetic screening, as the index case was the only affected individual from whom we had access to DNA). The c.3878A>G mutation predicts the amino acid substitution p.K1293R, affecting a highly conserved lysine within the NR2B C-terminus region, with a change from a medium-sized and polar (K) amino acid to a large and basic (R) amino acid, the arginine. This mutation, although not predicted to be damaging by in silico analysis, is located in a region that is highly conserved across species (Supplementary Fig. 2a–b), and was not detected in the remaining 496 subjects screened (including both patients and controls). Finally, no additional mutations in PSEN-1, PSEN-2 or APP genes were found in the patient (APOE ε3/ε3).

Single-marker association and haplotype analysis with AD

We likewise identified ten single nucleotide polymorphisms (SNPs) in the *GRIN2B*-coding region with no changes in the amino acid sequence, all in Hardy–Weinberg equilibrium. Five of these polymorphisms (rs34315573; rs1124894; rs35025065; rs1805522; rs45600931) were rare variants, present at a very low frequency (minor allele frequency = 1 %) and thus not included in our subsequent association study. Among the remaining common SNPs (rs7301328; rs1805482; rs3026160; rs1806201; rs1806191), four did not reach significance in our sample sets comparison (Table 2), even after stratification by age at disease onset or APOE ε4 carrier status (data not shown). By contrast as regards the last SNP, rs1806201, a significantly increased risk of developing AD was associated with the CT/TT genotypes compared with the CC genotype (Table 2). This result remained significant after inclusion of sex, age and APOE ε4-carrier status as covariates in logistic regression models. After stratification by APOE ε4 status (Table 3), the association between the rs1806201 T allele and AD was confirmed only among APOE ε4 non-carriers. However, the difference between ORs in the two strata was not subgroup significant ($p = 0.88$). Moreover, considering a frequency of the susceptibility allele of 0.2, disease prevalence of 0.07, and additive or dominant models of

Table 2 Genotypic and allelic frequencies of the *GRINB2* SNPs in AD patients and controls

SNP	Position		Controls (n = 226) (%)	Patients (n = 270) (%)	p value	p value[a]	OR (95 % CI)[a]
rs7301328	14018777	Genotype			0.40		
c.366C>G-Pro122		C/C	82 (36.3)	100 (37.0)			
		C/G	107 (47.3)	137 (50.7)			
		G/G	37 (16.4)	33 (12.2)			
		Allele			0.43	0.53	0.91 (0.69–1.21)
		C	271 (60.0)	337 (62.4)			
		G	181 (40.0)	203 (37.6)			
rs1805482	13764774	Genotype			0.20		
c.1665C>T-Ser555		C/C	96 (42.5)	104 (38.5)			
		C/T	102 (45.1)	117 (43.3)			
		T/T	28 (12.4)	49 (18.1)			
		Allele			0.12	0.33	1.15 (0.86–1.53)
		C	294 (65.0)	325 (60.2)			
		T	158 (35.0)	215 (39.8)			
rs3026160	13720043	Genotype			0.28[b]		
c.2514C>T-Cys838		C/C	178 (78.8)	227 (84.1)			
		C/T	45 (19.9)	41 (15.2)			
		T/T	3 (1.3)	2 (0.7)			
		Allele			0.12	0.36	0.80 (0.50–1.28)
		C	401 (88.7)	495 (91.7)			
		T	51 (11.3)	45 (8.3)			
rs1806201	13717508	Genotype			<0.001*		
c.2664 C>T-Thr888		C/C	143 (63.3)	122 (45.2)			
		C/T[c]	67 (29.6)	115 (42.6)			
		T/T[d]	16 (7.1)	33 (12.2)			
		Allele			<0.0001*	<0.001*	1.92 (1.40–2.63)
		C	353 (78.1)	359 (66.5)			
		T	99 (21.9)	181 (33.5)			
rs1806191	13716638	Genotype			0.86		
c.3534C>T-His1178		C/C	67 (29.6)	83 (30.7)			
		C/T	120 (53.1)	137 (50.7)			
		T/T	39 (17.3)	50 (18.5)			
		Allele			0.98	0.57	0.92 (0.70–1.22)
		C	254 (56.2)	303 (56.1)			
		T	198 (43.8)	237 (43.9)			

Basepair (bp) positions from current release of Ensembl, v73

OR odds ratio, *CI* confidence interval

* Statistically significant p values

[a] Values adjusted for sex, age and APOE-ε4 carrier status using logistic regression

[b] Fisher's exact test

[c] p value for heterozygotes <0.001; adjusted p value <0.01; adjusted OR = 1.96 (1.28–3.02)

[d] p value for homozygotes <0.01; adjusted p value <0.01; adjusted OR = 3.00 (1.49–6.04)

inheritance, our total sample and APOE ε4− subgroup had an 80 % or greater power to detect ORs as small as 1.53, whereas the APOE ε4+ subgroup appears not to be large enough to allow an adequately powered analysis (only ORs greater than 3.2 could be detected with an 80 % power).

Pairwise LD measures of the five SNPs are shown in Fig. 1. We observed marked differences in LD patterns between patients and controls. There is a region of high LD around the marker pair rs3026160–rs1806201 in patients, whereas this region has low LD in controls. One LD block, composed of

Table 3 *GRINB2* rs1806201 genotype and allele frequencies stratified according to APOE ε4 status

Subsample	Controls (%)	Patients (%)	p value	p value[a]	OR (95 % CI)[a]
APOE ε4−					
Genotype			0.001*		
C/C	131 (63.3)	69 (43.9)			
C/T	60 (29.0)	66 (42.0)			
T/T	16 (7.7)	22 (14.0)			
Allele			<0.001*	<0.001*	1.88 (1.35–2.64)
C	322 (77.8)	204 (65.0)			
T	92 (22.4)	110 (35.0)			
APOE ε4+					
Genotype			0.29[b]		
C/C	12 (63.2)	53 (46.9)			
C/T	7 (36.8)	49 (43.4)			
T/T	0 (0)	11 (9.7)			
Allele			0.10	0.11	2.09 (0.85–5.13)
C	31 (81.6)	155 (68.6)			
T	7 (18.4)	71 (31.4)			
			Breslow-Day $p = 0.88$		

OR odds ratio, *CI* confidence interval

* Statistically significant *p* values

[a] Values adjusted for sex and age using logistic regression

[b] Fisher's exact test

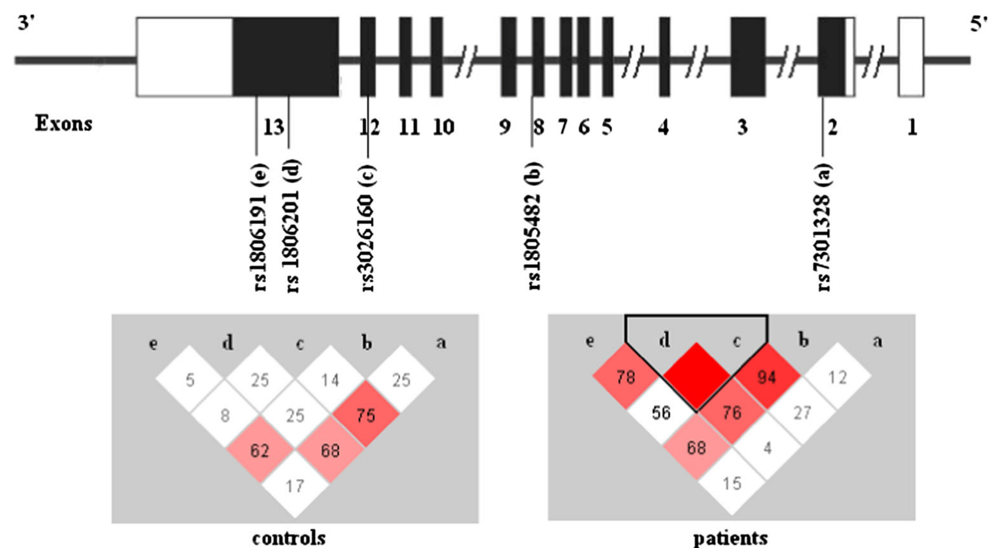

Fig. 1 Schematic representation of the GRIN2B gene and LD patterns in controls and patients. **a** *Black boxes* denote exons, *white boxes* denote untranslated regions. Because of their reverse orientation, 5′–3′ is read from right to left. **b** The intensity of the *box colour* corresponds to the strength of LD (D′/LOD). The numbers in each plot represent pairwise D' values × 100. The haplotype block was defined by the "solid spine of LD" (Haploview software)

these two SNPs, is generated by Haploview. All the markers studied except one (rs7301328) were present in the HapMap and 1,000 genomes databases and are part of different LD blocks both in CEU (North Americans of European ancestry) and TSI (Toscans in Italy) populations, with the exception of the rs1806201–rs1806191 pair. We then examined whether specific haplotypes of the *GRIN2B* gene increased the risk of developing AD. Interestingly, among the 17 five-marker inferred haplotypes with frequencies = 0.01 in at least one status group, one risk haplotype (CCCTC) and one protective haplotype (CCCCC) were identified (Table 4). Finally, regarding the APOE genotype frequencies, we observed that at least one ε4 allele increased AD risk by approximately sevenfold in a dose-dependent manner (data not shown). These findings emphasize that the well-established genetic factor APOE may also modify the overall risk of AD in our population.

Discussion

The alterations of glutamatergic synapses have been shown to be one of the earliest events and have long been considered the best pathological correlate of cognitive decline in AD. In this regard, prioritized attention is directed toward the NMDAR, particularly given their critical role in learning and memory and in view of the potential neuropathological role of these receptors' mediated

Table 4 Haplotype frequency estimation at the *GRIN2B* locus in controls and patients

5-SNP haplotype[a]	Estimated haplotype frequency		*p* value	*p* value[bc]	OR[b]
	Controls	Patients			
CCCTC	0.05	0.15	<0.000001*[d]	<0.0001*[d]	6.03
CCCCC	0.22	0.14	<0.001*[d]	<0.001*[d]	0.44
Global			<0.0000001*	<0.0001*	

OR odds ratio

* Statistically significant *p* values

[a] SNP order: rs7301328–rs1805482–rs3026160–rs1806201–rs1806191. Only individual haplotypes with frequencies ≥0.01 in at least one status group and with a significant *p* value are presented

[b] Values adjusted for sex, age and APOE-ε4 carrier status using logistic regression

[c] *p* values after 50,000 permutations

[d] Relative to each haplotype compared to all others ($n = 16$) pooled together

excitotoxicity in the evolution of AD. In this study, to investigate the hypothesis that *GRIN2B*, encoding the NMDA receptor subunit NR2B, represents a potential critical switch for genetic predisposition to AD, we performed the first extensive mutation analysis of this gene in a well-characterized cohort of patients with AD. We have for the first time to our knowledge identified a missense mutation in the coding regions of the *GRIN2B* that exist only in AD patients but not in controls, suggesting a close relationship between this pathological change of the postsynaptic NR2B-subunit and a selective alteration of synaptic structures in the brains of patients. In theory, it is difficult to predict whether this newly detected variant may have no apparent effect on the phenotype (benign polymorphisms) or may represent a pathogenic mutation underlying AD (no family members were available for analysis), supporting the interest of functional studies to assess the deleterious character of the mutation. In any case, the K1293R mutation map in the mechano-regulatory domain of NR2B (amino acids 1036–1433)), which is known to be the target of post-translational modifications, especially phosphorylation and cytoskeletal binding (Singh et al. 2012). Interestingly, this NR2B C-terminus region, in which the mutation is located, modulates the control mechanism found in many complex multi-subunit proteins, which ensures that only fully assembled and properly folded complexes reach the cell surface as functional receptors (Hawkins et al. 2004; Yang et al. 2007). It is reasonable to suggest that a mutation in this key regulatory domain, highly conserved in mammals and vertebrates, can affect the assembly mechanism itself by destabilizing the number and composition of extrasynaptically located dimers that predominantly contain NR2B during embryogenesis. On the other hand, it is not surprising that rare, damaging, heterozygous variants in the NMDARs genes may influence developmental expression patterns, reflecting the remodelling of native NMDARs in different neurodevelopmental human phenotypes (Metzler 2011). Indeed, from the clinical point of view, recent evidence strongly suggests that mutations in the prenatally already expressed *GRIN2B* gene leads to cognitive defects as the most consistent phenotypic feature in humans (Endele et al. 2010). In this view, experiments with knock-out mice expressing the homologous Nmdar2b gene, without the large intracellular C-terminal domain (Sprengel et al. 1998), display perinatal lethality for the homozygous −/− phenotype, as previously reported for the genetic ablation of NR2B (Kutsuwada et al. 1996). Hence, the modulation of NMDAR channel properties appears to be strongly dependent on the C-terminal domain of the NR2B subunit, which also reflects the non-functionality of the synaptic NMDAR-targeting system. Thus, the molecular data reported here argue that NR2B disregulation is likely to be a primary and pathogenic event, and emphasize the importance of future AD studies on the control of *GRIN2B* expression. In the second instance, we are the first to provide statistical evidence that multiple coding variants on the risk haplotype containing rs1806201, a very significant marker in our study, might play a role in mediating susceptibility to AD. On the other hand, the observed differences in LD patterns between patients and controls further support the possibility that risk and protective haplotypes exist. Despite being difficult to assign a direct action of this silent variant encoding also to the C-terminal domain, it became evident from other studies (Beste et al. 2010) that this SNP could have drastic functional effects by altering mRNA folding or stability and subsequent protein translation. This suggests that specific risk haplotypes or molecular variants of *GRIN2B* gene might provide an important clue to learn more about the molecular mechanisms underlying AD. Of particular interest are the APOE ε4 non-carriers, if we consider that over half of the cases (58.1 % in this study) do not carry this well-known predictor for AD risk. In our study, only this subgroup was associated with AD but the results obtained from the test of interaction show that no modifier effect can be ascribed to APOE ε4. The lack of association of the APOE ε4+ subgroup may be caused by low number of controls carrying this allele, resulting in insufficient power to detect significant differences. Altogether, the rs1806201 T allele might therefore be accountable for the inherited AD disease vulnerability, independent of the APOE genotype, at least in a Southern Italian population. It should be noted, however, that our study has some limitations that should be addressed. First, the sample size of screened subjects precludes us from making any definitive statements on the

associations between AD and the *GRIN2B* gene; as such, our findings should be considered preliminary, requiring further investigations to validate and more fully explain the associations we observed. Clearly, the analysis of a larger data set, to support or reject our findings, would be useful for the definitive confirmation of the results. Second, in our study of the role of *GRIN2B* in AD risk, we used the commonly identified SNPs in our investigation that did not include all representative SNPs in the entire gene. Some other rare functional SNPs, which may influence the susceptibility to AD, may have been missed and need to be investigated in more extensive independent replication populations. Third, our molecular screening over the coding sequence of *GRIN2B* did not identify any functional or non-synonymous polymorphisms, which therefore do not alter the amino acid sequence of the protein. However, these substitutions, rather than having a direct functional effect, may be in LD with genetic variants encompassing the non-coding, untranslated or regulatory regions of the *GRIN2B* gene that more likely could be associated with AD. More specifically, those substitutions located in regions of the 5′ flanking sequences, the 5′UTR, and all functional regions of this gene have not been systematically studied in AD. In the complex, all these variants could reflect the high selective pressure imposed on the coding sequence, taking into account that the human *GRIN2B* gene has 98 % overall amino acid sequence identity with mouse and rat sequences (Schito et al. 1997; Dorval et al. 2007). Finally, despite the ample in vitro and in vivo evidence, no data are available on the role of *GRIN2B* genetic variants in AD risk, and previous reports have given inconsistent and largely negative results (Seripa et al. 2008; Jiang and Jia 2009; Chen et al. 2010). Although, our preliminary findings do not constitute a direct replication of these initial studies (Jiang and Jia 2009; Chen et al. 2010) nor those involving sample sets of identical ethnicity (Seripa et al. 2008), they might plot a course not previously indicated in the direction of *GRIN2B*, and, specifically of the variation in the 3′ end of the gene, in susceptibility to disease. On the other hand, it should be emphasized that while in some cases these results could reflect genuine population differences, the presence of biological and genetic heterogeneity, population substructure, sample size, case selection, methodological and technical differences and study design could explain the discrepancies among studies. Concomitantly, our work also addresses another point: in this study, we found ten common and uncommon non-pathogenic variations in the *GRIN2B* gene. In particular, uncommon polymorphisms may also have important implications for genetic counselling in AD (Lleó et al. 2002). Recent evidence suggests that synonymous mutations observed at particular sites are under selection because they affect the thermodynamic stability of mRNA secondary structures (Chamary and Hurst 2005, 2009). Nevertheless, to what degree these mutations are favoured or opposed by selection due to their effects on mRNA stability is presently unclear. These results could therefore be used to estimate a simple and convenient way of measuring mutation rates, providing a parsimonious mechanism by which selection could act on synonymous sites. We are aware that our data should be-interpreted with caution; nevertheless, we believe that our findings represent the most thorough study yet performed on this gene for an AD-related phenotype. Particularly, our results provide further epidemiologic evidence that a Calabrian genetic peculiarity exists, essential in studies regarding genetically inherited and multifactorial disorders such as AD, and show that *GRIN2B* DNA testing is a powerful and sensitive tool for supporting the clinical diagnosis of this neurodegenerative disease. Clearly, more studies are required to enhance our understanding of NMDARs structure–function alteration relationships involved in the development of neurodegeneration and dementia. In terms of future work, it is important to detect new genetic risk profiles intersecting with the main pathogenic mechanisms potentially involved in AD, which may provide better therapeutic targets and therefore ensure new treatment strategies for this devastating disease.

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References

Andreoli V, Trecroci F, La Russa A, Cittadella R, Liguori M, Spadafora P, Caracciolo M, Di Palma G, Colica C, Gambardella A, Quattrone A (2011) Presenilin enhancer-2 gene: identification of a novel promoter mutation in a patient with early-onset familial Alzheimer's disease. Alzheimers Dement 7(6):574–578. doi:0.1016/.jalz.2011.2.010

Beste C, Baune BT, Domschke K, Falkenstein M, Konrad C (2010) Dissociable influences of NR2B-receptor related neural transmission on functions of distinct associative basal ganglia circuits. Neuroimage 52(1):309–315. doi:0.1016/.neuroimage.010.4.22

Bi H, Sze CI (2002) *N*-methyl-D-aspartate receptor subunit NR2A and NR2B messenger RNA levels are altered in the hippocampus and entorhinal cortex in Alzheimer's disease. J Neurol Sci 200(1–2):11–18. doi:0.1016/S0022-510X

Cacabelos R (2008) Pharmacogenomics in Alzheimer's disease. Methods Mol Biol 448:213–357. doi:10.1007/978-1-59745-205-2_10

Chamary JV, Hurst LD (2005) Evidence for selection on synonymous mutations affecting stability of mRNA secondary structure in mammals. Genome Biol 6(9):R75. doi:10.1186/gb-2005-6-9-r75

Chamary JV, Hurst LD (2009) The price of silent mutations. Sci Am 300(6):46–53

Chen C, Li X, Wang T, Wang HH, Fu Y, Zhang L, Xiao SF (2010) Association between NMDA receptor subunit 2b gene polymorphism and Alzheimer's disease in Chinese Han population in Shanghai. Neurosci Bull 26(5):395–400. doi:10.1007/s12264-010-0729-2

Crum RM, Anthony JC, Bassett SS, Folstein M (1993) Population-based norms for the Mini- Mental State Examination by age and educational level. JAMA 269(18):2386–2391. doi:10.1001/jama.1993.03500180078038

Cruts M, van Duijin CM, Backhovens H, Van den Broeck M, Wehnert A, Serneels S, Sherrington R, Hutton M, Hardy J, St George-Hyslop PH, Hofman A, Van Broeckhoven C (1998) Estimation of the genetic contribution of presenilin-1 and -2 mutations in a population-based study of presenile Alzheimer disease. Hum Mol Genet 7(1):43–51. doi:10.1093/hmg/7.1.43

Cull-Candy SG, Leszkiewicz DN (2004) Role of distinct NMDA receptor subtypes at central synapses. Sci STKE 2004(255):re16

Danysz W, Parsons CG (2012) Alzheimer's disease, β-amyloid, glutamate, NMDA receptors and memantine—searching for the connections. Br J Pharmacol 67(2):324–352. doi:10.1111/j.1476-5381.2012.02057

Darreh-Shori T, Siawesh M, Mousavi M, Andreasen N, Nordberg A (2012) Apolipoprotein ε4 modulates phenotype of butyrylcholinesterase in CSF of patients with Alzheimer's disease. J Alzheimers Dis 28(2):443–458. doi:10.3233/JAD-2011-111088

Dorval KM, Wigg KG, Crosbie J, Tannock R, Kennedy JL, Ickowicz A, Pathare T, Malone M, Schachar R, Barr CL (2007) Association of the glutamate receptor subunit gene GRIN2B with attention-deficit/hyperactivity disorder. Genes Brain Behav 6(5):444–452. doi:10.1111/j.1601-183X.2006.00273.x

Endele S, Rosenberger G, Geider K, Popp B, Tamer C, Stefanova I, Milh M, Kortüm F, Fritsch A, Pientka FK, Hellenbroich Y, Kalscheuer VM, Kohlhase J, Moog U, Rappold G, Rauch A, Ropers HH, von Spiczak S, Tönnies H, Villeneuve N, Villard L, Zabel B, Zenker M, Laube B, Reis A, Wieczorek D, Van Maldergem L, Kutsche K (2010) Mutations in GRIN2A and GRIN2B encoding regulatory subunits of NMDA receptors cause variable neurodevelopmental phenotypes. Nat Genet 2(11):1021–1026. doi:10.1038/ng.677

Frueh FW, Noyer-Weidner M (2003) The use of denaturing high-performance liquid chromatography (DHPLC) for the analysis of genetic variations: impact for diagnostic and pharmacogenetics. Clin Chem Lab Med 41(4):452–461. doi:10.15/CCLM.2003.068

Goate AM, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L et al (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349(6311):704–706. doi:10.1038/349704a0

Guerreiro RJ, Gustafson DR, Hardy J (2012) The genetic architecture of Alzheimer's disease: beyond APP, PSENs and APOE. Neurobiol Aging 33(3):437–456. doi:10.1016/j.neurobiolaging.2010.03.025

Hawkins LM, Prybylowski K, Chang K, Moussan C, Stephenson FA, Wenthold RJ (2004) Export from the endoplasmic reticulum of assembled *N*-Methyl-D-aspartic Acid receptors is controlled by a motif in the C terminus of the NR2 subunit. J Biol Chem 279(28):28903–28910. doi:10.1074/jbc.M402599200

Hu NW, Ondrejcak T, Rowan MJ (2012) Glutamate receptors in preclinical research on Alzheimer's disease: update on recent advances. Pharmacol Biochem Behav 100(4):855–862. doi:10.1016/j.pbb.2011.04.013

Jiang H, Jia J (2009) Association between NR2B subunit gene (GRIN2B) promoter polymorphisms and sporadic Alzheimer's disease in the North Chinese population. Neurosci Lett 450(3):356–360. doi:10.1016/j.neulet.2008.10.075

Kutsuwada T, Sakimura K, Manabe T, Takayama C, Katakura N, Kushiya E, Natsume R, Watanabe M, Inoue Y, Yagi T, Aizawa S, Arakawa M, Takahashi T, Nakamura Y, Mori H, Mishina M (1996) Impairment of suckling response, trigeminal neuronal pattern formation, and hippocampal LTD in NMDA receptor epsilon 2 subunit mutant mice. Neuron 16(2):333–344. doi:10.1016/S0896-6273(00)80051-3

Lau CG, Zukin RS (2007) NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat Rev Neurosci 8(6):413–426. doi:10.1038/nrn2153

Laube B, Hirai H, Sturgess M, Betz H, Kuhse J (1997) Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit. Neuron 18(3):493–503. doi:10.1016/S0896-6273(00)81249-0

Lleó A, Castellví M, Blesa R, Oliva R (2002) Uncommon polymorphism in the presenilin genes in familial Alzheimer's disease: not to be mistaken as a pathogenic mutation. Neurosci Lett 318(3):166–168. doi:10.1016/S0304-3940(01)02499-5

Luo L, Zhu Y, Xiong M (2012) A novel genome-information content-based statistic for genome-wide association analysis designed for next-generation sequencing data. J Comput Biol 19(6):731–744. doi:10.1089/cmb.2012.0035

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 34(7):939–944

Metzler M (2011) Mutations in NMDA receptors influence neurodevelopmental disorders causing epilepsy and intellectual disability. Clin Genet 79(3):219–220. doi:10.1111/j.1399-0004.2010.01610.x

Mony L, Kew JN, Gunthorpe MJ, Paoletti P (2009) Allosteric modulators of NR2B- containing NMDA receptors: molecular mechanisms and therapeutic potential. Br J Pharmacol 157(8):1301–1317. doi:10.1111/j.1476-5381.2009.00304.x

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256(5060):1217–1221. doi:10.1126/science.256.5060.1217

Olney JW, Wozniak DF, Farber NB (1997) Excitotoxic neurodegeneration in Alzheimer disease: new hypothesis and new therapeutic strategies. Arch Neurol 4(10):1234–1240. doi:10.1001/archneur.1997.00550220042012

Paoletti P (2011) Molecular basis of NMDA receptor functional diversity. Eur J Neurosci 33(8):1351–1365. doi:10.1111/j.1460-9568.2011.07628

Petralia RS (2012) Distribution of extrasynaptic NMDA receptors on neurons. Sci World J 2012:267120. doi:10.1100/2012/267120

Porsteinsson AP, Grossberg GT, Mintzer J, Olin JT, Memantine MEM-MD-12 Study Group (2008) Memantine treatment in patients) with mild to disease moderate Alzheimer's already receiving a cholinesterase inhibitor: a randomized, double-blind, placebo-controlled trial. Curr Alzheimer Res 5(1):83–89. doi:10.2174/5672058783884576

Reisberg B, Doody R, Stöffler A, Schmitt F, Ferris S, Möbius HJ, Memantine Study Group (2003) Memantine in moderate to-severe Alzheimer's disease. N Engl J Med 348(14):1333–1341. doi:10.1056/NEJMoa013128

Sadigh-Eteghad S, Talebi M, Farhoudi M (2012) Association of apolipoprotein E epsilon 4 allele with sporadic late onset Alzheimer's disease: a meta-analysis. Neurosciences (Riyadh) 17(4):321–326

Santangelo RM, Acker TM, Zimmerman SS, Katzman BM, Strong KL, Traynelis SF, Liotta DC (2012) Novel NMDA receptor

modulators: an update. Expert Opin Ther Pat 22(11):1337–1352. doi:10.1517/13543776.2012.728587

Schito AM, Pizzuti A, Di Maria E, Schenone A, Ratti A, Defferrari R, Bellone E, Mancardi GL, Ajmar F, Mandich P (1997) mRNA distribution in adult human brain of GRIN2B, a *N*-methyl-D-aspartate (NMDA) receptor subunit. Neurosci Lett 239(1):49–53. doi:0.1016/S0304-3940(97)00853-7

Seripa D, Matera MG, Franceschi M, Bizzarro A, Paris F, Cascavilla L, Rinaldi M, Panza F, Solfrizzi V, Daniele A, Masullo C, Dallapiccola B, Pilotto A (2008) Association analysis of GRIN2B, encoding *N*-methyl-D-aspartate receptor 2B subunit, and Alzheimer's disease. Dement Geriatr Cogn Disord 25(3):287–292. doi:10.1159/000118634

Singh P, Doshi S, Spaethling JM, Hockenberry AJ, Patel TP, Geddes-Klein DM, Lynch DR, Meaney DF (2012) N methyl-D-aspartate receptor mechanosensitivity is governed by C-terminus of NR2B subunit. J Biol Chem 287(6):4348–4359. doi:10.1074/jbc.M111.253740

Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, Greengard P (2005) Regulation of NMDA receptor trafficking by amyloid-β. Nat Neurosci 8(8):1051–1058. doi:10.1038/nn1503

Sprengel R, Suchanek B, Amico C, Brusa R, Burnashev N, Rozov A, Hvalby O, Jensen V, Paulsen O, Andersen P, Kim JJ, Thompson RF, Sun W, Webster LC, Grant SGN, Eilers J, Konnerth A, Li J, McNamara JO, Seeburg PH (1998) Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. Cell 92(2):279–289. doi:10.1016/S0092-8674(00)80921-6

Stein JL, Hua X, Morra JH, Lee S, Hibar DP, Ho AJ, Leow AD, Toga AW, Sul JH, Kang HM, Eskin E, Saykin AJ, Shen L, Foroud T, Pankratz N, Huentelman MJ, Craig DW, Gerber JD, Allen AN, Corneveaux JJ, Stephan DA, Webster J, DeChairo BM, Potkin SG, Jack CR Jr, Weiner MW, Thompson PM, Alzheimer's Disease Neuroimaging Initiative (2012) Genome-wide analysis reveals novel genes influencing temporal lobe structure with relevance to neurodegeneration in Alzheimer's disease. Neuroimage 51(2):542–554. doi:10.1016/j.neuroimage.2010.02.068

Sze CI, Bi H, Kleinschmidt-DeMasters BK, Filley CM, Martin LJ (2001) NMDA receptor subunit NR1, NR2A, and NR2B proteins and their phosphorylation status are altered selectively in Alzheimer's disease. J Neurol Sci 175(2):81–90. doi:10.1016/S0022-510X(00)00285-9

Tackenberg C, Grinschgl S, Trutzel A, Santuccione AC, Frey MC, Konietzko U, Grimm J, Brandt R, Nitsch RM (2013) NMDA receptor subunit composition determines beta-amyloid-induced neurodegeneration and synaptic loss. Cell Death Dis 25(4):e608. doi:10.1038/cddis.2013.129

Winblad B, Jones RW, Wirth Y, Stöffler A, Möbius HJ (2007) Memantine in moderate to severe Alzheimer's disease: a meta-analysis of randomised clinical trials. Dement Geriatr Cogn Disord 24(1):20–27. doi:10.1159/000102568

Yang W, Zheng C, Song Q, Yang X, Qiu S, Liu C, Chen Z, Duan S, Luo J (2007) A three amino acid tail following the TM4 region of the *N*-methyl-D-aspartate receptor (NR)2 subunits is sufficient to overcome endoplasmic reticulum retention of NR1-1a subunit. J Biol Chem 282(12):9269–9278. doi:10.1074/jbc.M700050200