Motoneuron-specific *NR3B* gene

No association with ALS and evidence for a common null allele

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ABSTRACT

Objective: The *GRIN3B* gene encodes NR3B, a motoneuron-specific member of the NMDA type of ionotropic glutamate receptors. NR3B reduces the Ca^{2+}-permeability as well as the overall current of the receptor response and may thereby protect motoneurons against glutamate-mediated excitotoxicity. We tested whether genetic dysfunction of *GRIN3B* is implicated in the pathogenesis of ALS.

Methods: We searched for mutations in the *GRIN3B* coding region (3.1 kb) in 117 individuals with familial ALS and in 46 individuals with sporadic ALS. We genotyped the newly identified *GRIN3B* null allele and four "tag single nucleotide polymorphisms (SNPs)" at the *GRIN3B* locus in 342 individuals with sporadic ALS and in 374 matched controls. The *GRIN3B* null allele frequency was determined in 2,128 individuals from a worldwide panel of 42 populations. We furthermore compared the *GRIN3B* coding sequence in primates (human–macaque) and rodents (rat–mouse) to evaluate the molecular evolution of *GRIN3B*.

Results: Thirty-two SNPs, including 16 previously unreported SNPs, one 27-bp deletion, a polymorphic CAG repeat, and a 4-bp insertion (insCGTT), were identified. Mutational and case–control studies did not reveal variants that cause or modify disease in ALS. Intriguing is an insCGTT variant that truncates the protein at its amino terminus and results in a *GRIN3B* null allele. We demonstrated a global distribution of the null allele with allele frequencies ranging between 0 and 0.38, and we delineated a null allele specific haplotype of 9.89 kb. Comparative genomic analysis across four taxa demonstrated accelerated evolution of NR3B in primates.

Conclusions: Our study supports the conclusions that 1) *GRIN3B* does not seem to be associated with familial or sporadic ALS, 2) the *GRIN3B* null allele is a common polymorphism, 3) the *GRIN3B* null allele has arisen once and early in human evolution, and 4) the *GRIN3B* gene belongs to a group of nervous system–related genes that have been subjected to faster evolution during evolution. ***Neurology*® 2007;▪▪:1–1**

ALS is a late-onset, progressive neurodegenerative disease selectively affecting motoneurons. Approximately 10% of cases are familial (FALS), of which 10% to 20% can be explained by mutations in *SOD1* and a smaller fraction by mutations in *Alsin*, *Senataxin*, *VABP*, and *Dynactin*.[1-6] Sporadic ALS (SALS) has been proposed to be a complex genetic disease,[7] but many of these studies were based on small numbers of individuals, and findings are conflicting. Hence, the pathogenesis of the majority of FALS and of practically all SALS cases is unknown.

Supplemental data at www.neurology.org

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Overactivation of glutamate receptors and increased calcium entry into the cell, referred to as excitotoxicity, has been postulated to contribute to motoneuron death in ALS.[8] The glutamate receptor subunit gene *GRIN3B* (MIM 606651; http://www.ncbi.nlm.nih.gov/Omim/)[9,10] encodes the NR3B subunit of the NMDA subclass of ionotropic glutamate receptors.[11] In mice, *Grin3b* is almost exclusively expressed in somatic motoneurons in brain stem and spinal cord.[10] A weaker signal intensity of *Grin3b* is found in motoneurons of the cranial nerve (III, IV, VI) nuclei, and in motoneurons of the lower spinal cord corresponding to Onuf's nucleus.[10] NR3B, when it coassembles with subunits NR1 and NR2, acts as an inhibitory subunit that decreases the current amplitude and Ca^{2+} permeability.[10,12] Therefore, NR3B may protect motoneurons against excitotoxicity, and dysfunction of NR3B may cause or predispose to motor neuron disease such as ALS. To test this idea, we performed an extensive genetic analysis of *GRIN3B* in ALS patients and normal individuals.

METHODS

Subjects. For all parts of this study, ALS patients were recruited after the diagnosis of definite or probable ALS was made according to the El Escorial criteria.[13] Informed consent was obtained from all individuals participating in this study. The study protocol was approved by the internal review board.

GRIN3B mutational analysis. The patient group was comprised of FALS patients (n = 117) and SALS patients (n = 45). All FALS individuals were from independent families that proved negative for mutations in *SOD1*. FALS individuals reported their ethnicity as white and of European descent. Control individuals (n = 374), matched for ethnicity, were either healthy volunteers or healthy spouses of patients.

GRIN3B case–control association study. The sample set included SALS patients (n = 342) and controls (n = 374). Sixty-two percent of SALS patients were men, and 38% were women. Age at onset and site of onset were determined by patients' report of first symptom(s) and were obtained in 86.5% and 85.3% of cases. Duration of disease was calculated as the time from clinical onset to death or to use of respiratory support. This information was available in 53.5% of SALS individuals because a substantial fraction of cases were recently recruited for this study and are still alive. Among all SALS patients, 74.8% had limb onset, and 25.2% had bulbar onset. Mean age at onset was 53.9 years (median 54.8), and the mean survival time was 47.4 months (median 39.0). All clinical data were in accordance with those reported in earlier studies. Control individuals consisted of either healthy volunteers or healthy spouses of patients and were matched to cases on the basis of age and sex. All SALS patients and control individuals were white, of European descent.

Human diversity panel. The frequency and ethnic distribution of the *GRIN3B* insCGTT variant was determined in a total of 2,128 individuals from 42 populations.[14,15]

Sequence analysis. *GRIN3B* exons 1 through 9 were amplified by PCR. The high GC content of the template (exon 1: 79%, exons 2 through 9: 69%) required inclusion of dimethylsulfoxide (DMSO) or betaine into all reactions. This was followed by direct sequencing of the PCR products in both directions on a Beckman Coulter CEQ 8800 Genetic Analysis System sequencer using the CEQ DTCS Quick Start Kit and DMSO/betaine. Sequence data were analyzed by use of Beckman CEQ 8000 software and assembled into contigs using Sequencher (version 4.2) software. At insertion/deletion polymorphisms, sequence analysis was performed manually, and sequencing primers to the sequence strand beyond the insertion/deletion were designed for resequencing. The complementary DNA (cDNA) numbering of sequence variations was derived from the human *GRIN3B* cDNA with adenine of the initiation codon numbered as 1 (GenBank accession number NM_138690).

Genotyping. Genotyping was performed using the fluorogenic 5′ nuclease technology/Taqman single nucleotide polymorphism (SNP) genotyping assays from Applied Biosystems (ABI). Four "tag SNPs" at the *GRIN3B* locus were selected with the Applied Biosystems SNP Browser 2.0, a software program for the selection of a minimum set of highly informative SNP markers by various "SNP tagging" methods. Among these methods, we have used the pairwise r^2 algorithm, a simple and widely used method for "SNP tagging."[16] We have used the pairwise r^2 method at a stringent threshold of 0.99 because low r^2 values typically result in loss of statistical power of a study and require increases in sample size.[17]

The "tag SNPs" were rs2301811, rs11669927, rs739883, and rs10414196 and are located on human chromosome 19p13.3 at the following positions (GenBank accession number NT_011255.14): rs2301811 at np 935578 in *WDR18* intron 1, rs11669927 at np 949798 upstream of *GRIN3B*, rs739883 at np 957768 in *GRIN3B* intron 3, and rs10414196 at np 971287 in *C10orf6* intron 1. Taqman assays for "tag SNPs" were ordered from Applied Biosystems as Taqman validated SNP genotyping assays.

Some sequence variants identified in the sequence analysis and the *GRIN3B* insCGTT variant were genotyped by use of Custom Taqman SNP genotyping assays from Applied Biosystems (ABI). Sequence files for Custom Taqman SNP genotyping assays were created following the manufacturer's guidelines. The position of the *GRIN3B* insCGTT variant (rs10666583) is in *GRIN3B* exon 3 (between np 955896 and np 955897). SNP genotyping was performed using 2 ng DNA, AmpliTaq Gold DNA polymerase (Applied Biosystems) with an annealing temperature of 60 °C for 40 cycles on a thermocycler. The Taqman assays were read and analyzed with an ABI Prism 7900HT sequence detection system in up to 374 controls.

Statistical analysis. ***Association study.*** Using a significance level of 0.05 and a power of 80%, our study would detect risk alleles at an allele frequency of 10% with a rela-

tive risk of 1.75 or greater. Case–control analysis was conducted by analyzing the χ^2 distribution of *GRIN3B* alleles and genotypes in a 2×2 contingency table. FINETTI software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was applied to analyze marker genotypes for Hardy–Weinberg equilibrium using the Pearson χ^2 test statistic, the likelihood ratio χ^2 test statistic, and an Exact test.

Haplotype analysis. SNPHAP software (http://www.gene.cimr.cam.ac.uk/clayton/software) was applied to the five marker data in both groups of the association study to predict haplotypes. Only data sets with at least four genotyped markers were considered for haplotype construction. Haplotype frequencies were calculated from haplotypes directly assigned to individuals using the Bayesian approach of SNPHAP. All haplotypes with frequencies of <0.01 were lumped into one class. p values were calculated from the χ^2 distribution of the n haplotypes in a $2 \times n$ contingency table.

LD analysis. To determine the linkage disequilibrium (LD) structure at the *GRIN3B* locus, we calculated the LD measure D′ between every marker pair in the five-marker set, using Haploview software (http://www.broad.mit.edu/mpg/haploview/index.php).[18] We also used Haploview to generate a graphical representation of the LD matrix.

RESULTS Mutational analysis of *GRIN3B* in FALS and detection of a common null allele. We performed an extensive mutational analysis of the *GRIN3B* protein coding exons 1 through 9 in 45 FALS individuals. In addition, we screened the 1,033-bp exon 3 of *GRIN3B*, encoding the putative ligand-binding and channel-forming domains, in a second group of 72 FALS cases unrelated to the first group of FALS individuals. In most of the FALS cases, linkage data were not available or were inconclusive due to small family sizes. To account for autosomal recessive inheritance and low-penetrant mutations, we furthermore tested *GRIN3B* protein coding exons 1 through 9 in 46 individuals with SALS.

Our mutational analysis in 117 FALS and 46 SALS cases identified a total of 35 sequence variations distributed across the *GRIN3B* coding region of 3,129 bp, except no variants were found in exons 4, 5, and 6 (table 1 and figure E-1 on the *Neurology* Web site at www.neurology.org). A graphical representation of all sequence variations across the *GRIN3B* gene, using the genotype viewer software (VG2; http://pga.gs.washington.edu/VG2.html), among the 163 individuals (117 FALS, 46 SALS) is shown in figure E-1A. Thirty-two sequence variations were single base changes that, on an average, occurred every 98 bp of coding sequence. This level of DNA sequence variation is higher than reported.[19-21] Sixteen of the 32 nucleotide substitutions were nonsynonymous changes that did not affect amino acids highly conserved among different family members of glutamate receptors (data not shown). Only three (R486Q, T577L, A845T) of these 16 missense variants occurred within functional domains, i.e., the two lobules S1 and S2 forming the ligand-binding domain and the four membrane-associated regions M1 through M4 comprising the channel (figure 1).[22,23] These functional domains approximately start at P415 and end at L850 of NR3B by comparison with the crystallized structure of NR1 ligand-binding domain and hydrophobicity profile.[22]

Despite these evolutionary constraints, we detected a 4-bp insertion (c.1396-1397insCGTT) in codon 465 of exon 3. This insertion is predicted to lead to out-of-frame translation and a premature stop codon with the first nucleotide at c.1447 in exon 3. As a result, a truncated protein of 482 amino acids (including a novel peptide of 17 amino acids) instead of 1,043 amino acids of the mature protein is generated. Because this putative protein lacks all four membrane-associated regions, including the second domain forming the channel pore and S2 lobule of ligand-binding domain, a null allele is the most likely result of this variant. Interestingly, among the 163 individuals (117 FALS, 46 SALS) tested for exon 3, 60 (55%) were heterozygous and 12 (11%) were homozygous for the 4-bp insertion.

Exon 9 of *GRIN3B* contains a polyglutamine-encoding CAG-repeat motif which we found repeated six or seven times. We furthermore identified an in-frame 27-bp deletion in exon 9 (c.3082-3108del) predicted to result in a protein lacking nine amino acids at the C-terminal domain before reaching the first base of the termination codon at c.3130. Cross-species comparison of NR3B does not reveal similarity of the region where the 27-bp deletion resides and demonstrates reduction of homology after glycine at amino acid position 890 (figure E-2A). Alignment of the *GRIN3B* amino acid sequence with other glutamate receptor subunits does not show homology of the region surrounding the 27-bp deletion, furthermore indicating that the 27-bp deletion is unlikely to be of functional significance (figure E-2B).

Among these 35 sequence alterations observed in *GRIN3B*, 16 of the 32 single bases changes, the CAG repeat polymorphism, and the 27-bp deletion were previously not reported as validated/nonvalidated SNPs in public databases (table 1). Among these newly identified variants, all were present in 241 tested controls with the exception of five single base changes: c.552C>T (P184P), c.1209A>G (A403A), c.1686G>A (T562T), c.1698C>T (I566I), and 1457G>A (R486Q). Fur-

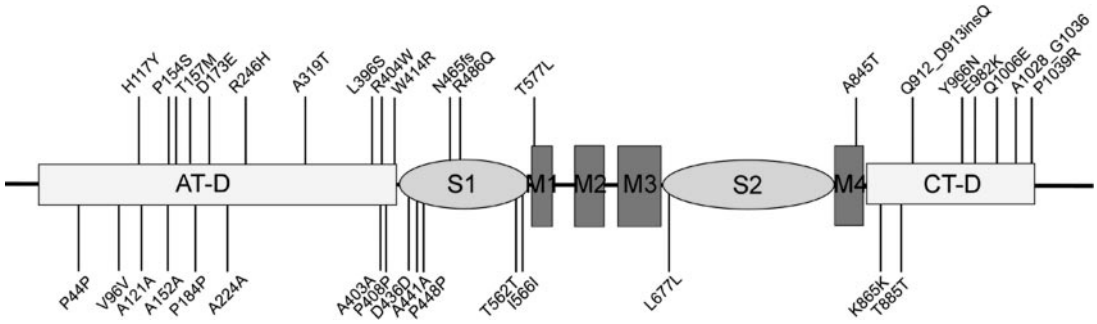
Table 1 Sequence variations identified in *GRIN3B*

Nucleotide change	Amino acid change	Exon	FALS (n = 45)*		SALS (n = 45)		Allele frequency	dbSNP	Contr	Affected relative
			Het	Homo	Het	Homo				
Substitution										
Synonymous										
c.132C>A	P44P	1	12	18	13	14	0.494		+	
c.288G>A	V96V	1	1	0	3	0	0.022		+	
c.363G>C	A121A	1	14	22	16	23	0.667	rs2301813	+	
c.456C>T	A152A	2	9	0	4	0	0.072		+	
c.552C>T	P184P	2	0	0	1	0	0.006		–	
c.672G>A	A224A	2	6	0	1	0	0.039		+	
c.1209A>G‡	A403A‡	3	3	0	1	0	0.012		–	–
c.1224A>G	P408P	3	56	13	19	6	0.347	rs11880849†	+	
c.1308C>T	D436D	3	20	0	12	0	0.098		+	
c.1323G>A	A441A	3	46	13	21	3	0.304	rs4806908	+	
c.1344T>C	P448P	3	55	15	20	5	0.353	rs4806909	+	
c.1686G>A	T562T	3	3	0	1	0	0.012		–	–
c.1698C>T	I566I	3	1	0	0	0	0.003		–	
c.2031G>C	L677L	3	50	14	19	4	0.322	rs12973948†	ND	
c.2595G>A	K865K	7	1	0	0	0	0.006		+	
c.2655G>C	T885T	8	10	2	5	8	0.194	rs2285907	+	
Nonsynonymous										
c.349C>A	H117Y	1	12	15	13	13	0.450	rs12986002†	+	
c.460C>T	P154S	2	3	0	2	0	0.028		+	
c.470C>T	T157M	2	16	2	13	0	0.183	rs2240154	+	
c.519C>A	D173E	2	8	0	1	0	0.050		+	
c.737G>A	R246H	2	5	0	9	0	0.078		+	
c.955G>A	A319T	2	4	0	2	0	0.033		+	
c.1187T>C	L396S	3	8	1	0	1	0.037	rs12978900†	+	
c.1210C>T‡	R404W‡	3	62	34	22	12	0.540	rs4807399	+	
c.1240T>C	W414R	3	47	7	18	5	0.273	rs2240157	+	
c.1457G>A	R486Q	3	0	0	1	0	0.003		–	
c.1730C>T	T577M	3	55	14	22	4	0.628	rs2240158	+	
c.2533G>A	A845T	7	13	2	6	6	0.194	rs2285906	+	
c.T2896T>A	Y966N	9	25	7	21	8	0.422	rs10417824†	+	
c.2944G>A	E982K	9	0	0	3	0	0.017		+	
c.3016C>G	Q1006E	9	22	14	23	15	0.572	rs10401245†	+	
c.3116C>G	P1039R	9	14	4	11	9	0.283	rs10401454†	+	
Insertion/Deletion										
c.1396_1397 insCGTT	N465fs	3	41	10	19	2	0.467	rs10666583†	+	
c.2736_2737 insCAG	Q912_D913insQ	9	7	0	13	0	0.111		+	
c.3081-3108 del27nt	A1028_G1036del	9	11	1	16	2	0.183		+	

* For *GRIN3B* exon 3, familial ALS (FALS) (n = 117).
† Nonvalidated single nucleotide polymorphism.
‡c.1209A>G, position 3 of codon triplet GCA; c.1210C>T, position 1 of codon triplet CGG.
Contr = control; SALS = sporadic ALS; het = heterozygous; homo = homozygous; + = presence; – = absence; ND = not determined.

Functional domains of NR3B are shown as predicted for ionotropic glutamate receptors. S1 and S2 lobes form the ligand-binding domain; M1, M2, M3, and M4 are membrane-associated domains; AT-D and CT-D are the amino-terminal and carboxyl-terminal domains.

Figure 1 Position of nonsynonymous sequence variations in *GRIN3B* showing bias toward occurrence outside of functional domains of the NR3B receptor subunit



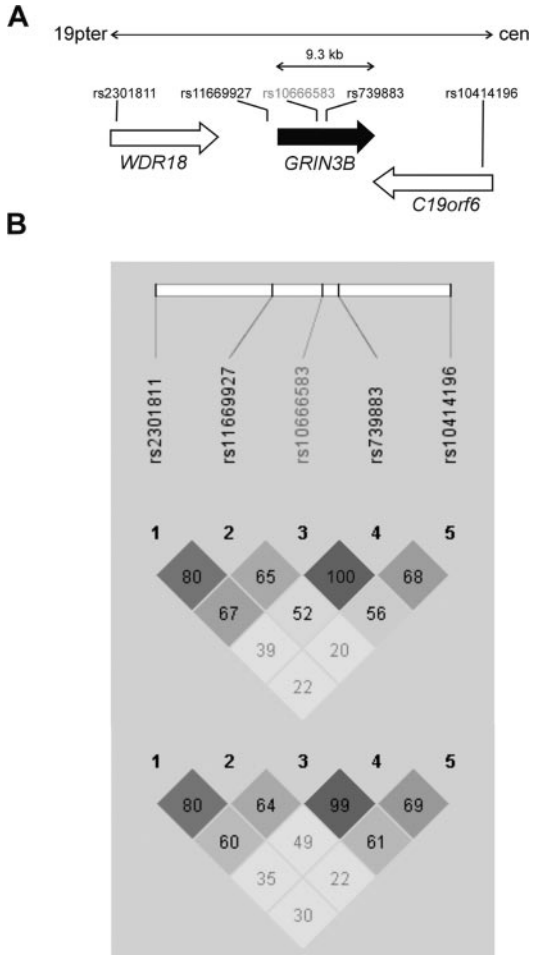
thermore, all variants reported as nonvalidated SNPs at dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) were verified in controls. Sequence variants c.1209A>G (A403A) and c.1686G>A (T562T) were present jointly in each of three FALS cases (RB0410, RB3010, and RB1321, marked with * in figure E-1A) and in one SALS case (RB1197, marked with *) but in none of the other cases. We analyzed an affected relative of FALS individual RB3010 and did not detect the two variants, indicating that these changes do not cause ALS. The variants c.552C>T (P184P) and c.1698C>T (I566I), found in one FALS and one SALS case, are both synonymous changes and do not map to splice site consensus sequences, suggesting that these variants are not disease-causing mutations. The remaining sequence was a coding change (c.1457C>A, R486Q) that occurred in one SALS individual (RB3332, marked with ** in figure E-1A) but was not present in 374 controls tested by SNP genotyping. This variant alters a residue located at the surface of the extracellular ligand-binding domain facing the outside of protein. The corresponding amino acid in both rat and mouse NR3A is Q.

To assess the functional impact of the R486Q and the c.1396_1397insCGTT variants, we investigated the electrophysiologic properties of the NMDA receptor complex by cotransfecting HEK293 cells with constructs expressing mutant NR3B in combination with constructs expressing NR1 and NR2A subunits (figure E-3; see E-Methods for details about cell transfection and electrophysiology). The current response in cells coexpressing NR3B was significantly smaller than those without NR3B, confirming previous results by others and us.[10,12] The amount of suppression of response was comparable with both NR3B wild-type and NR3B-R486Q. In contrast, the suppression was not observed in cells expressing the c.1396_1397insCGTT variant. From these facts, we deemed the R486Q change does not have a significant functional consequence on NR3B, whereas the c.1396_1397insCGTT mutation functionally leads to a null mutant.

Overall, our mutational analysis identified a wide spectrum of sequence variations in the coding sequence of *GRIN3B*, none of which seem to be of direct etiologic significance for monogenic ALS. The frequent observation of the 4-bp insertion (c.1396_1397insCGTT) in patients and controls is striking and supports two conclusions. First, the *GRIN3B* insCGTT variant is a common polymorphism in humans. Second, lack of NR3B in individuals homozygous for *GRIN3B* null allele, suggested to increase the glutamate-mediated response of the NMDA receptor, does not cause motor neuron disease.

Assessment of *GRIN3B* null allele as a susceptibility factor for ALS. The common occurrence of the *GRIN3B* null allele in ALS and control individuals prompted us to study this variant in more detail. Although not disease-causing by itself, the *GRIN3B* null allele may increase the risk for ALS and act as a susceptibility factor. We therefore determined the frequency of the *GRIN3B* insCGTT variant in a group of patients with SALS and control individuals, both of European ancestry. To explore the contribution of the *GRIN3B* locus to the development and course of disease in general, we included a set of highly informative SNPs ("tag SNPs") in our association study to capture the genetic variation at the *GRIN3B* locus (figure 2A). We compared allele and genotype frequencies of the five markers in 342 SALS cases and in 374 controls. There was no deviation of *GRIN3B* genotypes from Hardy–Weinberg equilibrium. The allele frequency of the *GRIN3B* null allele was nearly identical in both groups (0.294 in 342 cases and 0.296 in 374 controls), and genotype distributions did not vary significantly (table 2). At marker rs739883, genotype frequencies were different in affected and unaffected individuals

Figure 2 Genomic and linkage disequilibrium structure at the *GRIN3B* locus



(A) Flanking known genes and genotyped SNP/Indel markers at the *GRIN3B* locus. The four "tag single nucleotide polymorphisms (SNPs)" are black; the *GRIN3B* null allele (insCGTT) is light gray. (B) Pairwise linkage disequilibrium (LD) between markers genotyped in 342 individuals with sporadic ALS (upper panel) and in 374 controls (lower panel). LD was measured with D′ and visualized using the Haploview program.[18] D′ values are shown in the boxes. There is only weak LD at the *GRIN3B* locus.

($p = 0.034$) but were below significance when corrected for multiple testing. For all other markers, allele or genotype frequencies were not significantly associated with disease. There was no evidence for *GRIN3B* allele/genotype frequency variation when SALS cases were grouped into limb onset patients and bulbar onset patients (table 2).

Twelve common haplotypes (frequency ≥ 0.01) were predicted by SNPHAP software for the five-marker set data from both the cases and the controls (table E-1). The association between haplotype and disease status was not significant. In addition, to determine the extent of linkage disequilibrium across *GRIN3B*, we calculated pairwise linkage disequilibrium, measured by D′, between the five markers using Haploview software.[18] This analysis revealed that the *GRIN3B* gene is not contained in a larger haplotype block (figure 2B).

Assessment of *GRIN3B* null allele as a modifier gene for ALS. To establish the potential role of *GRIN3B* as a modifier gene for ALS, we compared the clinical course of the SALS patients with and without the insCGTT allele. We also assessed whether certain genotypes of the other four markers are associated with ALS disease characteristics. There was no association between *GRIN3B* allele/genotype frequency distribution and site of onset (table 3). Analysis of variance did not show significant differences between the *GRIN3B* genotypes and clinical phenotypes such as mean age at onset and duration of disease (table 4).

Assessment of the *GRIN3B* frequency distribution in a worldwide panel of human populations. Might the high frequency of the null allele in Europeans be the result of evolutionary selective pressures? Loss of gene function has been proposed to be a common evolutionary mechanism in which populations adapt to evolutionary selective pressures.[24] Thus, if the *GRIN3B* null allele has been positively selected for under certain environmental circumstances, this may be reflected by a peculiar geographic distribution of this variant. We have therefore investigated the frequency and ethnic distribution of the *GRIN3B* insCGTT variant in a total of 2,128 individuals from 42 different populations from around the world.[14,15] The *GRIN3B* null allele showed a global distribution and was observed in 39 of 42 populations (table E-2). A graphical presentation of the geographic distribution and descriptions of the population samples can be found at http://alfred.med.yale.edu. The *GRIN3B* insCGTT allele was not detected in only three isolated populations. In all other populations, *GRIN3B* insCGTT was detected at allele frequencies ranging between 0.01 and 0.38. In Western European populations, *GRIN3B* insCGTT was observed with allele frequencies between 0.24 and 0.29, closely aligning with the allele frequency of 0.296 seen in our controls (table 2). The global distribution of the *GRIN3B* insCGTT variant shows a pattern that has been observed at other loci (e.g., SI001466R, SI001049O, SI000139N; http://alfred.med.yale.edu). The F_{st} value for the *GRIN3B* insCGTT variant, 0.094, is also not unusual. The F_{st} value for the insCGTT variant is well within one SD of the mean F_{st} for a very large number of essentially random SNPs studied on the same 42

Table 2 Association of ALS with *GRIN3B* genotypes/alleles

Location	dbSNP	Group (n)	No. (frequency) of genotype or allele						
			Genotype			*p* Value	Allele		*p* Value
WDR18 intron 1	rs2301811		GG	GT	TT		G	T	
		Cases (333)	75 (22.5)	170 (51.1)	88 (26.4)	0.930	320 (48.0)	346 (52.0)	0.778
		Limb (212)	46 (21.7)	110 (51.9)	56 (26.4)	0.907	202 (47.6)	222 (52.4)	0.758
		Bulbar (70)	18 (25.7)	35 (50)	17 (24.3)	0.759	71 (50.7)	69 (49.3)	0.908
		Controls (350)	78 (22.3)	175 (50.0)	97 (27.7)		331 (47.3)	369 (52.7)	
upstream GRIN3B	rs11669927		CC	CT	TT		C	T	
		Cases (334)	43 (20.6)	109 (52.2)	57 (27.3)	0.241	319 (47.8)	349 (52.2)	0.769
		Limb (209)	43 (20.6)	109 (52.2)	57 (27.3)	0.303	195 (46.7)	223 (53.3)	0.539
		Bulbar (71)	19 (26.8)	38 (53.5)	14 (19.7)	0.305	76 (53.5)	66 (46.5)	0.281
		Controls (345)	88 (25.5)	159 (46.1)	98 (28.4)		335 (48.6)	355 (51.4)	
GRIN3B exon 3	rs10666583		—/—	—/CGTT	CGTT/CGTT		—	CGTT	
		Cases (332)	162 (48.8)	145 (43.7)	25 (7.5)	0.200	469 (70.6)	195 (29.4)	0.938
		Limb (210)	102 (48.6)	91 (43.3)	17 (8.1)	0.385	295 (70.2)	125 (29.8)	0.942
		Bulbar (68)	36 (52.9)	27 (39.7)	5 (7.4)	0.730	99 (72.8)	37 (27.2)	0.580
		Controls (362)	186 (51.4)	138 (38.1)	38 (10.5)		510 (70.4)	214 (29.6)	
GRIN3B intron 3	rs739883		CC	CT	TT		C	T	
		Cases (335)	37 (11.0)	176 (52.5)	122 (36.4)	0.034	250 (37.3)	420 (62.7)	0.693
		Limb (209)	21 (10.0)	111 (53.1)	77 (36.8)	0.044	153 (36.6)	265 (63.4)	0.915
		Bulbar (71)	9 (12.7)	32 (45.1)	30 (42.3)	0.878	50 (35.2)	92 (64.8)	0.808
		Controls (350)	52 (14.9)	150 (42.9)	148 (42.3)		254 (36.3)	446 (63.7)	
C10orf6 intron1	rs10414196		AA	AC	CC		A	C	
		Cases (338)	107 (31.7)	176 (52.1)	55 (16.3)	0.717	390 (57.7)	286 (42.3)	0.669
		Limb (212)	68 (32.1)	109 (51.4)	35 (16.5)	0.830	265 (57.8)	153 (42.2)	0.686
		Bulbar (71)	23 (32.4)	35 (49.3)	13 (18.3)	0.992	92 (57.0)	50 (43.0)	0.915
		Controls (351)	111 (31.6)	175 (49.9)	65 (18.5)		397 (56.6)	305 (43.4)	

populations, providing evidence against any selection operating on this polymorphism. Hence, the *GRIN3B* null allele frequency distribution seems to be the result of random genetic drift and demography rather than the effect of selection events.

Delineation of a *GRIN3B* null allele specific haplotype. The *GRIN3B* null allele might have arisen to its high frequency through strong selection on a gene variant in its surroundings. Thus, the *GRIN3B* null allele would represent a molecular imprint of a selection event on a nearby locus. To address this question and to evaluate the evolutionary history of the *GRIN3B* null allele, we have studied the allelic pattern of polymorphisms flanking the *GRIN3B* null allele. The *GRIN3B* insCGTT allele was present in 3 of the 12 common haplotypes predicted by SNPHAP, i.e., on TTinsCA, GCinsCA, and TTinsCC (table E-1). The haplotypes furthermore revealed that all individuals homozygous for insCGTT variant were also homozygous at rs739883, located in *GRIN3B* intron 3, whereas only a fraction showed homozygosity at rs2301811, rs11669927, and rs10414196 flanking *GRIN3B* (data not shown). This indicates that recombination has occurred at both ends of *GRIN3B*. Distally, the *GRIN3B* null allele haplotype can be further narrowed down by investigating the genotypes of polymorphisms identified in the mutational analysis (figure E-1). Individuals RB0841, RB1237, RB1238, RB3481, and RB4144 (marked with *** in figure E-1), which are homozygous carriers of the 4-bp insertion, show homozygosity for all other 13 markers in exon 3, all 8 markers in exon 2, and for all 4 markers in exon 1. The homozygous presence of

Table 3 Association between *GRIN3B* genotypes/alleles and site of onset

		No. (frequency) of genotype or allele						
dbSNP	Site onset (n)	Genotype			*p* Value	Allele		*p* Value
rs2301811		GG	GT	TT		G	T	
	Limb (212)	46 (21.7)	110 (51.9)	56 (26.4)	0.78	202 (47.6)	222 (52.4)	0.53
	Bulbar (70)	18 (25.7)	35 (50)	17 (24.3)		71 (50.7)	69 (49.3)	
rs11669927		CC	CT	TT		C	T	
	Limb (209)	43 (20.6)	109 (52.2)	57 (27.3)	0.35	195 (46.7)	223 (53.3)	0.16
	Bulbar (71)	19 (26.8)	38 (53.5)	14 (19.7)		76 (53.5)	66 (46.5)	
rs10666583		—/—	—/CGTT	CGTT/CGTT		—	CGTT	
	Limb (210)	102 (48.6)	91 (43.3)	17 (8.1)	0.82	295 (70.2)	125 (29.8)	0.57
	Bulbar (68)	36 (52.9)	27 (39.7)	5 (7.4)		99 (72.8)	37 (27.2)	
rs739883		CC	CT	TT		C	T	
	Limb (209)	21 (10.0)	111 (53.1)	77 (36.8)	0.49	153 (36.6)	265 (63.4)	0.16
	Bulbar (71)	9 (12.7)	32 (45.1)	30 (42.3)		50 (35.2)	92 (64.8)	
rs10414196		AA	AC	CC		A	C	
	Limb (212)	68 (32.1)	109 (51.4)	35 (16.5)	0.93	245 (57.8)	179 (42.2)	0.88
	Bulbar (71)	23 (32.4)	35 (49.3)	13 (18.3)		81 (57.0)	61 (43.0)	

these markers clearly defines a null allele specific haplotype constructed from genotypes of individuals all of which were of European descent. Although other populations were not studied for haplotypes to prove the uniqueness of a null allele specific haplotype, the nature of the mutation as a 4-bp insertion makes independent occurrence on a different genetic background less likely than recurrence of a single–base pair change. One may therefore speculate that the 4-bp insertion has occurred only once in evolution.

At rs2285906 (2533G>A), located in exon 7, individuals RB1237, RB1238, RB3481, and RB4144 are homozygous for the (common) "G" allele, whereas RB0841 is heterozygous. Therefore, rs2285906 flanks the null allele specific haplotype distally.

Taken together, the null allele specific haplotype has been interrupted by recombination events on both sites of the *GRIN3B* gene, making "genetic hitchhiking" of a nearby locus unlikely. Delineation of the *GRIN3B* null allele haplotype to an interval of only 9.89 kb, flanked proximally by rs11669927 and distally by rs2285906, indicates that the insertion of 4 bp has occurred early in human evolution. The global distribution of the null allele furthermore indicates that the 4-bp insertion has arisen before the expansion out of Africa. The absence of long-range LD around the 4-bp insertion allele suggests that the relatively high frequency of the deletion allele is achieved through genetic drift and demography rather than positive selection but does not yet completely rule out the possibility of selection.

We note that selection could have occurred on a null allele that is ancient and resides on a haplotype that has been broken down by recombinations.

Molecular evolution of the *GRIN3B* gene in different mammals. Recently, it was demonstrated that genes implicated in various aspects of the nervous system show accelerated rates of evolution during human evolution.[25] The coding sequences of these genes were compared across four mammalian species (human, macaque, rat, and mouse), and the K_a/K_s ratio, i.e., the ratio of nonsynonymous substitution rate (K_a) to synonymous substitution rate (K_s), was calculated separately for primates (human–macaque) and rodents (rat–mouse). By the same approach, we measured the primate–rodent K_a/K_s ratios to determine whether the NR3B protein has evolved faster in primates (see E-Methods for details about calculation of evolutionary parameters). The K_a, K_s, and K_a/K_s values were 0.026, 0.101, and 0.258 for the primate comparison, respectively, and 0.023, 0.204, and 0.112 for the rodent comparison. This disparity between the two ratios was $p = 0.0005$. In agreement with earlier studies, the observation of a higher K_a/K_s ratio in primates argues that *GRIN3B* belongs to the group of nervous system–related genes that show accelerated evolution in the lineage leading to humans.

DISCUSSION In this study, we revealed a high

Table 4 Analysis of variance between *GRIN3B* genotypes and mean age of onset, mean disease duration

SNP	Subject characteristics	No. (frequency) of genotype			*p* Value
rs2301811		GG	GT	TT	
	Cases, n	66 (22.7)	149 (51.2)	76 (26.1)	
	Mean age at onset, years	53.2 ± 12.0	53.4 ± 14.3	55.0 ± 14.2	0.65
	Cases, n	40 (22.6)	91 (51.4)	46 (26.0)	
	Mean duration, months	52.7 ± 32.1	46.0 ± 32.1	43.6 ± 27.4	0.37
rs11669927		CC	CT	TT	
	Cases, n	65 (22.3)	149 (51.2)	77 (26.5)	
	Mean age at onset, years	53.1 ± 12.1	54.0 ± 14.4	54.2 ± 13.8	0.87
	Cases, n	41 (23.3)	85 (48.3)	50 (28.4)	
	Mean duration, months	48.2 ± 27.0	48.9 ± 34.6	41.1 ± 26.4	0.34
rs10666583		—/—	—/CGTT	CGTT/CGTT	
	Cases, n	145 (50.3)	121 (42.0)	22 (7.6)	
	Mean age at onset, years	54.4 ± 13.2	53.1 ± 14.3	56.0 ± 14.4	0.59
	Cases, n	95 (54.3)	68 (38.9)	12 (6.9)	
	Mean duration, months	44.7 ± 28.7	48.1 ± 31.7	54.8 ± 44.6	0.51
rs739883		CC	CT	TT	
	Cases, n	30 (10.3)	150 (51.5)	111 (38.1)	
	Mean age at onset, years	55.1 ± 13.8	52.0 ± 14.5	55.9 ± 12.4	0.07
	Cases, n	20 (11.3)	88 (49.7)	69 (39.0)	
	Mean duration, months	48.1 ± 38.1	46.7 ± 30.7	45.8 ± 29.0	0.96
rs10414196		AA	AC	CC	
	Cases, n	95 (32.3)	150 (51.0)	49 (16.7)	
	Mean age at onset, years	54.1 ± 13.4	53.4 ± 14.1	55.0 ± 13.4	0.78
	Cases, n	59 (32.8)	91 (50.6)	30 (16.7)	
	Mean duration, months	46.6 ± 29.6	47.6 ± 33.4	43.0 ± 25.5	0.78

Age at onset and mean duration are given as mean ± SD.

degree of genetic diversity of *GRIN3B* among humans, including a null allele that exists at high frequency and occurs worldwide. This diversity does not by itself cause impairment of apparent motoneuronal function leading to a disease or increased susceptibility for ALS. Our discovery of complete NR3B deficiency in approximately 10% of the normal population in the absence of any obvious clinical phenotype is intriguing considering the postulated function of the NR3B subunit to protect motoneurons against excitotoxic injury. The high prevalence of NR3B deficiency may suggest that NR3B is functionally redundant and that its absence can be compensated for by other proteins. Given the lack of a candidate for such a compensatory role, this possibility seems rather unlikely because NR3A is only weakly expressed in motoneurons.[26]

It is conceivable that the requirement for NR3B function has been lost in humans and that *GRIN3B* is degenerating in the human lineage. Mutations that lead to loss of function are numerous and occur far more often than mutations that confer a novel function to a protein. In fact, gene loss is a common motif of molecular evolution and may, as adaptive loss of function, represent a dynamic evolutionary response to changes in selective pressures.[24] According to this model, the human *GRIN3B* gene has acquired an inactivating mutation that is spreading through the human population to become common or reach fixation. *GRIN3B*, functional in other species, may be in the process of becoming a pseudogene in humans. Even without evolutionary constraints on NR3B function, the deficiency of NR3B could still exert subtle effects on neuromuscular function or could modify the course of disorders of the motoneuron other than ALS.

A possibility how a neutral *GRIN3B* null allele could have reached the high allele frequency is because of positive selection on a polymorphism in a locus closely linked to the null allele. Thus, the evolutionary favored hypothetical variant would "drag" the neutral *GRIN3B* null allele ("genetic

hitchhiking"). Haplotype construction in individuals homozygous for the null allele using genotypes of polymorphisms in and around *GRIN3B* narrowed the haplotype to an interval of only 9.89 kb. This null allele specific haplotype spans only part of *GRIN3B*, defined by critical recombinations in the 5′ region and in exon 7, and is therefore unlikely to encompass other loci.

Alternatively, absence of NR3B function may confer an advantage to individuals under certain evolutionary selective pressures.[27] Gene variants that have been positively selected may show striking differences in allele frequencies in different populations.[28] We determined the geographic variation in *GRIN3B* null allele frequencies in a human diversity panel and found worldwide occurrence of the null allele. The null allele was present at a strikingly different distribution and allele frequencies ranging between 0 and 0.38, but similar patterns of genetic variation have been observed in other polymorphisms. We therefore conclude that the *GRIN3B* null allele has spread by neutral genetic drift and represents the result of demographic history rather than a selection event.

A key role of NR3B for motoneuron function has recently been reported in *Grin3b* knock-out mice.[29] Mice homozygous for the *Grin3b* null allele develop progressive paresis of the extremities and die at approximately P5. Histologic analysis in these animals has demonstrated loss of motoneurons in the spinal cord.

The explanation for the discrepancy of our results with the phenotype observed in *Grin3b* knock-out mice is currently unknown. This awaits further characterization of the knock-out animal, which is only published in abstract form.[29]

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