VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death

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Amyotrophic lateral sclerosis (ALS) is an incurable degenerative disorder of motoneurons. We recently reported that reduced expression of *Vegfa* causes ALS-like motoneuron degeneration in *Vegfa*^{Δ/Δ} mice. In a meta-analysis of over 900 individuals from Sweden and over 1,000 individuals from Belgium and England, we now report that subjects homozygous with respect to the haplotypes -2,578A/-1,154A/-634G or -2,578A/-1,154G/-634G in the *VEGF* promoter/leader sequence had a 1.8 times greater risk of ALS ($P = 0.00004$). These 'at-risk' haplotypes lowered circulating VEGF levels *in vivo* and reduced *VEGF* gene transcription, IRES-mediated *VEGF* expression and translation of a novel large-VEGF isoform (L-VEGF) *in vivo*. Moreover, *SOD1*^{G93A} mice crossbred with *Vegfa*^{Δ/Δ} mice died earlier due to more severe motoneuron degeneration. *Vegfa*^{Δ/Δ} mice were unusually susceptible to persistent paralysis after spinal cord ischemia, and treatment with Vegfa protected mice against ischemic motoneuron death. These findings indicate that *VEGF* is a modifier of motoneuron degeneration in human ALS and unveil a therapeutic potential of Vegfa for stressed motoneurons in mice.

ALS, also called Lou Gehrig or Charcot disease, is a devastating untreatable neurodegenerative disorder with a yearly incidence of 1–2 per 100,000 (refs. 1–5). It is characterized by progressive wasting and weakness of limb, bulbar and respiratory muscles due to degeneration of motoneurons in the spinal cord, brain stem and motor cortex. Disease onset is usually in the fifth or sixth decade of life and, in most affected individuals, progresses to death due to respiratory failure 3–5 years after onset. There are unexplained large individual differences in clinical presentation (initial symptoms may be spinal or bulbar in nature), age of onset (ranging from juvenile to the very elderly) and survival time (from a few months to more than 20 years). Elucidating the modifying factors has important consequences for our understanding of neuron degeneration and development of therapy.

Approximately 5–10% of ALS is familial^{6,7}. Five types of familial ALS (FALS) have been assigned to loci of the human genome^{6,8}. ALS1, an autosomal dominant form of adult ALS affecting 14–23% of individuals with FALS, is associated with more than 100 mutations in the gene encoding Cu/Zn superoxide dismutase (*SOD1*) on chromosome 21q22.1–2. Recently, the gene associated with ALS2, an autosomal recessive form of juvenile ALS, was reported to encode a protein containing domains with homology to Ran/Rho-GEFs⁹. Dysfunction of dynactin 1-mediated axonal transport can also lead to human motoneuron disease. The genetic causes of the other forms of FALS are not yet known. The etiology and pathogenesis of the more common sporadic form of the disease are poorly understood^{1–4,10}. Besides a family history of ALS, age and male gender are the only established risk factors for ALS. Sporadic ALS (SALS) is

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believed to be a multi-factorial disease in which modifying genes and environmental agents affect its clinical manifestation, but few associated genes have so far been identified^{7,11–16}.

Using Cre/loxP-mediated gene targeting to delete the hypoxia-response element (HRE) in the promoter region of the gene encoding vascular endothelial growth factor in mice (*Vegfa*^{tm2Pec}, herein called *Vegfa*^Δ), we recently showed that impaired expression of *Vegfa* predisposed *Vegfa*^{Δ/Δ} mice to adult-onset progressive motoneuron degeneration, with many neuropathological and clinical signs reminiscent of human ALS^{17,18}. It was not clear, however, whether *VEGF* is also involved in the pathogenesis of human ALS. Here, we show that *VEGF* is a modifier gene associated with motoneuron degeneration in individuals with ALS and in a mouse model of ALS. In addition, we provide evidence that treatment of mice with *Vegfa* stimulates motoneuron survival in stressed conditions.

RESULTS

Analysis of the HRE, *HIF1A* and *EPAS1* in individuals with ALS

A targeted deletion of the HRE in the *Vegfa* promoter impaired hypoxic upregulation of the expression of *Vegfa* and caused adult-onset motoneuron degeneration in *Vegfa*^{Δ/Δ} mice¹⁷. We therefore analyzed whether variations in the HRE sequence confer greater risk for ALS in humans. We found no sequence alterations in individuals with SALS or FALS from Sweden ($n = 250$), Belgium ($n = 96$) or England ($n = 94$). Because hypoxic upregulation of VEGF occurs through binding of the *HIF1A* and *EPAS1* (also called *HIF2A*) transcription

complexes to the HRE region, we also screened 96 individuals with SALS and 200 unaffected individuals from Sweden for sequence variations in *HIF1A* and *EPAS1*. We did not detect any exonic sequence variations causing functionally relevant missense mutations in *HIF1A* or *EPAS1* (data not shown). We then examined whether single-nucleotide polymorphisms (SNPs) in *VEGF* might confer a greater risk for motoneuron degeneration in individuals with ALS. Because both baseline and hypoxia-induced *Vegfa* expression levels were reduced in *Vegfa*^{Δ/Δ} mice¹⁷, we tested whether SNPs downregulating expression of *VEGF* might increase the susceptibility to ALS. We analyzed variations in the promoter or 5' untranslated region (UTR) of *VEGF*, which previously have been documented to downregulate *VEGF* expression: 2,578C→A (ref. 19), 1,154G→A (ref. 19) and 634G→C (ref. 20; Fig. 1a).

VEGF variations and ALS in the Swedish population

We initially genotyped 349 individuals with SALS and FALS and 275 unrelated healthy controls of similar age, geography and ethnicity drawn randomly from a population-based prospective study cohort from northern Sweden (Table 1). None of the genotype frequencies in the control population deviated from Hardy–Weinberg equilibrium (Table 2). We found no differences between affected individuals and controls in the distribution of the –2,578C→A ($P = 0.41$) or –634G→C ($P = 0.61$) variations, but the –1,154A allele was more prevalent in affected individuals than controls (allele frequency of 0.33 in affected individuals versus 0.28 in controls; $P = 0.029$). Individuals

Figure 1 Genetic association of *VEGF* with ALS.

(a) Structure of *VEGF*. The SNPs in the promoter and 5' UTR, the ATG codon of the secreted VEGF isoforms (only VEGF¹⁸⁹ is shown) and the CTG1 codon of L-VEGF are indicated. Cleavage of L-VEGF after the signal peptide (blue arrow) generates the secreted VEGF forms. (b) Median VEGF plasma levels in individuals with SALS and spouses from Sweden. Differences were calculated between the indicated genotypes and all others (Mann–Whitney U-test) in the spouses (^a $P = 0.13$, ^b $P = 0.17$, ^c $P = 0.08$) and in the affected individuals (^d $P = 0.016$, ^e $P = 0.008$, ^f $P = 0.005$). VEGF levels were compared between affected individuals and spouses of the indicated genotypes (^g $P < 0.001$, ^h $P = 0.26$, ⁱ $P = 0.011$, ^j $P = 0.008$). (c,d) Models of the predicted two-dimensional structure of the 5' UTR containing the common –634G (c) or variant –634C (d) allele. The structure of the 5' leader sequence of both alleles is comparable, except in the core region of the IRES-B sequence upstream of the CUG1 start codon of L-VEGF, which is substantially remodeled. (e) Western blot of L-VEGF and VEGF¹⁸⁹ in extracts of COS cells transfected with a *VEGF* expression cassette containing the entire 5' UTR in-frame with the cDNA encoding the VEGF¹⁸⁹ isoform. (f) Correlation of median VEGF serum values with the different genotypes in the Belgian control population. Statistical differences were calculated by comparing the indicated genotypes with all other genotypes (Mann–Whitney U-test; ^a $P = 0.02$, ^b $P = 0.017$, ^c $P = 0.0005$). (g) Meta-analysis of the ALS risk (ORs and 95% c.i.) associated with the AAG/AAG and AGG/AGG genotypes (combined) in individual and pooled populations. Breslow–Day homogeneity test showed no heterogeneity between the observed ORs in the different subpopulations ($P = 0.32$).

Table 1 Characteristics of study populations

Study populations	Total	Male/female	Age at onset ^a	Survival ^b	Spinal onset	Bulbar onset
Sweden						
SALS	292	165/127	64 (20–90)	^c	202	90
FALS	57	29/28	62 (10–81)	^c	45	12
Population-based controls	275	138/137	60 (50–75)	–	–	–
Healthy spouses	106	56/50	66 (32–95)	–	–	–
Neurological controls	173	92/81	67 (32–88)	–	–	–
Belgium						
SALS	153	97/56	59 (18–84)	33 (27–39)	115	38
Healthy controls	426	204/222	54 (19–95)	–	–	–
England (Birmingham)						
SALS	90	57/33	61 (16–87)	30 (25–35)	74	16
Healthy spouses	96	26/70	60 (28–84)	–	–	–
England (London)						
SALS	158	99/59	58 (26–79)	38 (31–46)	106	52
Healthy controls	143	84/59	58 (27–88)	–	–	–

^aMedian age (with lowest and highest values in parentheses) at onset for individuals with ALS and at blood sampling for controls. ^bKaplan–Meier analysis was used to estimate the median (95% c.i.) survival in months after diagnosis. ^cSurvival of deceased Swedish individuals with ALS was measured in years: individuals survived for <4 years (76% of SALS; 77% of FALS), 4–10 years (20% of SALS; 18% of FALS) or >10 years (4% of SALS; 5% of FALS).

Of the Swedish individuals with SALS and FALS, Belgian individuals with SALS and Birmingham and London individuals with SALS, 92%, 68%, 65%, 40% and 76%, respectively, had died and 8%, 23%, 17%, 60% and 16%, respectively, were censored at the time of study; the others were lost for analysis.

carrying the at-risk A allele had a greater risk for ALS than those carrying the G allele (odds ratio (OR) = 1.3; 95% confidence interval (c.i.) = 1.0–1.7). When comparing genotypes at position –1,154, we found that A/A but not G/A conferred an additional risk for ALS relative to G/G (OR = 2.4; $P = 0.006$ for A/A; OR = 0.9; $P = 0.76$ for G/A), indicating that the at-risk effect of the A allele was recessive. Overall, a greater fraction of affected individuals than controls was A/A homozygous (13% versus 6%; $P = 0.001$; recessive model).

VEGF variations in FALS and SALS in the Swedish population

The Swedish ALS cohort consisted of 292 individuals with SALS and 57 with FALS without mutations in *SOD1* (Table 1). To assess whether the observed association was true for FALS as well as for SALS, we stratified our sample accordingly. The –1,154A allele frequency was similar in individuals with FALS and with SALS (0.35 and 0.33, respectively) and was higher in both than in the controls (0.28). When comparing genotypes at position –1,154, only A/A conferred

Table 2 Association of variations in VEGF and individuals with ALS in Sweden

Genotype	Controls	Number (frequency; $n = 349$)	P value	Individuals with ALS		
	Number (frequency; $n = 275$)			Crude OR (95% c.i.)	Logistic OR (95% c.i.)	P value ^a
–2,578C/C	70 (0.25)	88 (0.25)	0.39 ^b	0.9 (0.6–1.4)	0.9 (0.6–1.4)	0.72
–2,578C/A	148 (0.54)	173 (0.50)				
–2,578A/A	57 (0.21)	88 (0.25)				
–2,578C	288 (0.52)	349 (0.50)	0.41 ^c	1.1 (0.9–1.4)		
–2,578A	262 (0.48)	349 (0.50)				
–1,154G/G	138 (0.50)	162 (0.46)				
–1,154G/A	122 (0.44)	141 (0.40)	0.001 ^d	2.6 (1.4–4.9)	2.4 (1.3–4.6)	0.006
–1,154A/A	15 (0.06)	46 (0.13)				
–1,154G	398 (0.72)	465 (0.67)				
–1,154A	152 (0.28)	233 (0.33)	0.029 ^c	1.3 (1.0–1.7)	0.9 (0.7–1.3)	0.72
–634G/G	145 (0.53)	179 (0.51)				
–634G/C	108 (0.39)	138 (0.40)				
–634C/C	22 (0.08)	32 (0.09)	0.86 ^b	0.9 (0.5–1.6)	0.8 (0.4–1.5)	0.44
–634G	398 (0.72)	496 (0.71)				
–634C	152 (0.28)	202 (0.29)				

^a P values of ORs (logistic regression) were calculated after adjustment for gender and age. ^b P values were calculated using the χ^2 contingency test with 2 d.f. ^c P values were calculated using the χ^2 contingency test with 1 d.f. ^dCalculation of ORs for the –1,154G→A SNP indicated a recessive model. Therefore the P value under the assumption of a recessive model is given.

Distribution of genotypes (frequencies) for the indicated SNPs in individuals with ALS and controls. ORs were calculated relative to genotypes –2,578C/C, –1,154G/G or –634C/C or alleles –2,578C, –1,154G or –634C.

Table 3 Standardized pairwise maximum-likelihood linkage disequilibria (D') for individuals with SALS and controls

	Belgium		Sweden		Birmingham		London	
	-1,154G/A	-634G/C	-1,154G/A	-634G/C	-1,154G/A	-634G/C	-1,154G/A	-634G/C
-2,578C/A	0.999/0.935	-0.999/-0.969	0.974/0.999	-0.940/-0.944	0.966/0.999	-0.999/-0.950	0.978/0.955	-0.999/-0.856
-1,154G/A		-0.999/-0.968		-0.999/-0.999		-0.999/-0.999		-0.999/-0.913

an additional risk for FALS relative to G/G (OR = 3.4; P = 0.005) and for SALS (OR = 2.5; P = 0.006). Individuals homozygous with respect to the -1,154A allele were less prevalent among controls (6%) than among individuals with FALS or SALS (18%, P = 0.001, and 12%, P = 0.004, respectively; recessive model). To extend our findings, we included two additional control populations with similar age and gender distribution and drawn from the same geographic and ethnic region. The first population, consisting of 173 individuals admitted to the hospital for neurological disorders distinct from ALS, served as neurological controls to examine whether the A allele was specifically associated with ALS and not with any other neurological disorder. The second population consisted of 106 spouses of individuals with ALS. The observed -1,154A allele frequency in the populations was 0.27 and 0.26, respectively, similar to that in the initial control population. In the remaining analysis, we focused our attention on the population with SALS, because its pathogenesis is unknown and because only individuals with SALS were available in populations from other areas that we examined (see below).

VEGF haplotypes and SALS in the Swedish population

We also determined haplotypes, because susceptibility to complex diseases is often attributable to multiple alleles whose collective effects may be more readily predicted by determining the full haplotype information. Pairwise maximum-likelihood analysis of linkage disequilibrium (D') showed a high degree of linkage disequilibrium among the SNPs (Table 3). We then inferred haplotypes at positions -2,578, -1,154 and -634 by the maximum-likelihood method and compared their frequencies in individuals with SALS versus controls (Supplementary Table 1 online). Only four of the eight possible three-loci haplotypes occurred at an appreciable frequency. Of those, haplotype -2,578A/-1,154A/-634G (hereafter called haplotype AAG) was more prevalent in affected individuals than controls (0.33 versus 0.26; P = 0.006), indicating that it is the at-risk haplotype for the Swedish population. We confirmed the four common haplotypes using independent methods (Supplementary Table 2 online). When we translated genotypes into haplotype combinations, we found that only individuals who were homozygous with respect to haplotype AAG (AAG/AAG) had significantly greater risk for SALS (OR = 2.0; c.i. = 1.2–3.2; P = 0.007). Swedish individuals who were homozygous with respect to haplotype -2,578A/-1,154G/-634G (AGG/AGG) also had greater risk (OR = 1.3). This difference did not reach significance because of insufficient power but became significant in the meta-analysis (see below).

Low VEGF plasma levels in at-risk individuals with ALS

The analysis in *Vegfa*^{Δ/Δ} mice indicated that low levels of Vegfa cause motoneuron degeneration¹⁷. VEGF plasma levels were also ~50% lower in individuals with SALS (median value 39 pg ml⁻¹; n = 195) than in unaffected spouses (71 pg ml⁻¹; n = 105; P < 0.0001 after adjustment for age and gender). VEGF plasma levels did not correlate with age at onset, with bulbar versus spinal onset or with progression of disease. Within each group (individuals with SALS and unaffected

spouses), VEGF plasma levels were lowest in individuals carrying the at-risk AAG/AAG or AGG/AGG genotypes, but VEGF levels were always lower in affected individuals than in controls of the same genotype (Fig. 1b). VEGF levels were also lowest in Belgian individuals carrying the AAG/AAG or AGG/AGG genotypes (see below). Thus, low VEGF plasma levels are associated with the genotypes conferring the greatest risk for ALS. The small sample size of individuals with FALS who were homozygous with respect to the AAG and AGG haplotypes (n = 6) precluded meaningful correlation of VEGF plasma levels with these at-risk genotypes.

Low VEGF expression by at-risk alleles

To confirm that the at-risk alleles did not merely statistically associate with but instead functionally affected VEGF expression, we analyzed whether the at-risk alleles reduced VEGF gene expression *in vitro*. Given the strong linkage disequilibrium between the three documented SNPs, we examined their effect in a haplotype context. We cloned a luciferase reporter gene cassette downstream of the VEGF promoter and 5' UTR containing either the at-risk haplotype AAG or AGG or a control haplotype CGC (the counterpart of the AAG haplotype) and used it to transfect glioma cells, which we then exposed to normoxia or hypoxia. Univariate analysis showed significant differences between the three different constructs (P < 0.001). Pairwise comparison showed that the luciferase activity levels in normoxia were 41% lower for haplotype AAG (P = 0.002) and 30% lower for haplotype AGG (P = 0.01) relative to haplotype CGC. In hypoxia, luciferase activities were 35% lower for haplotype AAG (P < 0.001) and 43% lower for haplotype AGG (P < 0.001). There were no significant differences between the AAG and AGG constructs (P = 0.78). Thus, the at-risk SNPs, in a haplotype context, lower VEGF expression *in vitro*.

To examine in more detail how each of the individual SNPs influenced VEGF gene expression, we evaluated their effect separately. We were unable to assess the transcriptional activity of the -2,578C→A variation, because an isolated -2,469 to -2,714 nucleotide promoter fragment did not enhance the expression of a CMV promoter-luciferase reporter construct above background levels (data not shown). This finding does not exclude the possibility that the -2,578C→A variation influences VEGF expression in the context of the entire promoter. To assess the -1,154G→A variation, we cloned a fragment of 185 nucleotides in front of the luciferase gene. Compared with the -1,154G allele, luciferase expression levels by the proximal VEGF promoter containing the -1,154A allele were 25% lower in normoxia (P < 0.001) and 26% lower in hypoxia (P < 0.001).

The -634G→C variation is located in the 5' UTR, which is known to regulate VEGF expression at the posttranscriptional level^{21,22}. Because little is known as to how deregulated posttranscriptional control may contribute to disease phenotypes, however, we assessed whether the at-risk -634G allele affected the activity of internal ribosomal entry site B (IRES-B). This element is involved in enhancing translation initiation of VEGF at the classical AUG²¹ and regulates the production of a large VEGF (L-VEGF) isoform, the translation of

which is initiated at an alternative CUG1 codon²² (Fig. 1a). Analysis of the secondary structure, predicted by the mfold 2.3 folding procedure²³, of the entire 5' UTR leader sequence indicated that the change -634G→C, for comparable stabilities ($\Delta G = -468 \text{ kCal mol}^{-1}$), did not affect the main part of the leader structure but substantially remodeled the region upstream of the CUG1, which is the core region of the IRES-B activity (Fig. 1c,d). To evaluate the translational activity of IRES-B, we transfected glioma cells with a bicistronic reporter plasmid. Compared with the -634C allele, relative IRES-B activity with the -634G allele was 16% lower in normoxia ($P = 0.025$) and 23% lower in hypoxia ($P < 0.001$). The difference was more pronounced in hypoxia because IRES-B-driven gene expression is increased under hypoxic conditions by the -634C allele ($P = 0.024$) but not by the -634G allele ($P = 0.28$). We determined the production of L-VEGF by transfection of an expression cassette containing the entire 5' UTR (including the CUG1 start codon of L-VEGF at -540) in-frame with the cDNA encoding the matrix-attached VEGF¹⁸⁹ isoform²² (Fig. 1e). Densitometric quantification of the 43-kDa L-VEGF bands in cell extracts after immunoblotting showed that, relative to the -634C allele, production of L-VEGF with the -634G allele was 20% lower in normoxia ($P < 0.001$; Fig. 1e) and also 20% lower in hypoxia ($P = 0.03$). Because L-VEGF is cleaved to form 23-kDa VEGF²², levels of the latter were also 20% lower with the -634G allele ($P = 0.004$), providing further confirmation of the functional involvement of this allele.

VEGF and SALS in additional populations

In an attempt to replicate the association of low VEGF-producing genotypes with SALS in other populations, we genotyped individuals with SALS of Belgian and British origin (Table 1). We analyzed two British populations, one from Birmingham and the other from the greater London area, and matched control individuals and those with SALS for age, ethnic and geographic distributions. Genotype

frequencies of the -2,578C→A, -1,154G→A and -634G→C variations in all controls were in full accordance with Hardy-Weinberg equilibrium (Table 4). In the Belgian population, more affected individuals than controls carried the -2,578A/A genotype (26% versus 20%; $P = 0.042$; recessive model; Table 4), and the -1,154A/A genotype was also more prevalent in affected individuals (10% versus 8%; $P = 0.24$; recessive model). The -634G allele was more prevalent in affected individuals than controls (0.69 versus 0.61; $P = 0.009$), and individuals who were homozygous with respect to haplotype AGG had a 5.1 times greater risk for SALS ($P = 0.006$; Table 5). In the Birmingham population, more individuals with SALS than controls carried the -2,578A/A genotype (30% versus 19%; $P = 0.024$; recessive model) and the -1,154A/A genotype (19% versus 6%; $P = 0.005$; recessive model). The -634G allele was also more prevalent in affected individuals than controls (0.73 versus 0.62; $P = 0.013$), and individuals carrying the AAG/AAG genotype had a 3.5 times greater risk for ALS ($P = 0.006$; Table 5). In the greater London population, none of the alleles was individually associated with SALS, but individuals who were homozygous with respect to haplotype AGG had a 1.7 times greater risk (Table 5). Thus, at the genotype level, we found an association for the -1,154A allele and for the -1,154A/A genotype in multiple populations (significantly in the Swedish and Birmingham populations but insignificantly in the Belgian population) and for the -634G allele in the Belgian population. At the haplotype level, individuals carrying the AAG/AAG genotype had greater risk for ALS in the Swedish and British (Birmingham) populations, whereas individuals with the AGG/AGG genotype had greater risk for ALS in the Belgian and British (London) populations (though the difference in this latter population was not significant).

Unfortunately, no blood samples were available from the Belgian and British individuals with ALS to correlate VEGF levels with at-risk genotypes for ALS. Nonetheless, in an effort to replicate our observation that at-risk genotypes were associated with low VEGF

Table 4 Association of variations in VEGF and individuals with SALS

Genotype	Belgium			England (Birmingham)			England (London)		
	Control	SALS		Control	SALS		Control	SALS	
	Number (frequency; $n = 426$)	Number (frequency; $n = 153$)	P value	Number (frequency; $n = 96$)	Number (frequency; $n = 90$)	P value	Number (frequency; $n = 143$)	Number (frequency; $n = 158$)	P value
-2,578C/C	127 (0.30)	34 (0.22)		20 (0.20)	21 (0.23)		43 (0.30)	48 (0.30)	
-2,578C/A	216 (0.51)	79 (0.52)		57 (0.59)	42 (0.47)		66 (0.46)	68 (0.43)	
-2,578A/A	83 (0.20)	40 (0.26)	0.042 ^a	19 (0.19)	27 (0.30)	0.024 ^a	34 (0.24)	42 (0.27)	0.29 ^a
-2,578C	470 (0.55)	147 (0.48)		97 (0.51)	84 (0.47)		152 (0.53)	164 (0.52)	
-2,578A	382 (0.45)	159 (0.52)	0.016 ^a	95 (0.49)	96 (0.53)	0.23 ^a	134 (0.47)	152 (0.48)	0.34 ^a
-1,154G/G	205 (0.48)	68 (0.44)		42 (0.44)	36 (0.40)		66 (0.46)	77 (0.49)	
-1,154G/A	187 (0.44)	70 (0.46)		48 (0.50)	37 (0.41)		58 (0.41)	64 (0.41)	
-1,154A/A	34 (0.08)	15 (0.10)	0.24 ^a	6 (0.06)	17 (0.19)	0.005 ^a	19 (0.13)	17 (0.11)	0.50 ^a
-1,154G	597 (0.70)	206 (0.67)		132 (0.69)	109 (0.61)		190 (0.66)	218 (0.69)	
-1,154A	255 (0.30)	100 (0.33)	0.19 ^a	60 (0.31)	71 (0.39)	0.049 ^a	96 (0.34)	98 (0.31)	0.50 ^a
-634G/G	162 (0.38)	71 (0.46)		34 (0.35)	48 (0.53)		67 (0.47)	76 (0.48)	
-634G/C	199 (0.47)	69 (0.45)		51 (0.53)	35 (0.39)		60 (0.42)	68 (0.43)	
-634C/C	65 (0.15)	13 (0.09)	0.027 ^b	11 (0.11)	7 (0.08)	0.024 ^b	16 (0.11)	14 (0.09)	0.40 ^b
-634G	523 (0.61)	211 (0.69)		119 (0.62)	131 (0.73)		194 (0.68)	220 (0.70)	
-634C	329 (0.39)	95 (0.31)	0.009 ^a	73 (0.38)	49 (0.27)	0.013 ^a	82 (0.32)	96 (0.30)	0.43 ^a

^a P values were calculated using the χ^2 contingency test with 1 d.f. for alleles and genotypes under a recessive model. ^b P values were calculated using the χ^2 contingency test with 2 d.f.

expression in the Swedish population, we quantified VEGF serum levels in 295 control individuals from Belgium. Similar to the Swedish population, VEGF levels were lowest in individuals who were homozygous with respect to haplotypes AAG and AGG, and we observed the most significant association when comparing genotypes AAG/AAG and AGG/AGG together, relative to all other genotypes ($P = 0.0005$; **Fig. 1f**).

At-risk genotypes in pooled study populations

It is not uncommon for the same haplotype to be associated with different disease susceptibility in distinct populations. Pooling distinct study populations has, therefore, been proposed to provide a better estimate of the overall risk²⁴. We pooled the genotypes of affected and control individuals for meta-analysis and assessed the risk of SALS after adjusting for the population of origin by logistic regression. The overall risk for SALS was 1.4 times greater with the -2,578A/A genotype (c.i. = 1.1–1.8; $P = 0.006$; recessive model), 1.5 times greater with the -1,154A/A genotype (c.i. = 1.1–2.0; $P = 0.012$; recessive model) and 1.2 times greater with the -634G/G genotype (c.i. = 1.0–1.5; $P = 0.034$; recessive model). We also evaluated the risk for individuals who were homo- or heterozygous with respect to the various haplotypes in the meta-analysis, after assessing that a single overall logistic regression of their distribution, when accounting simultaneously for the country of origin, was significant ($P = 0.046$).

The overall risk for SALS was 1.6 times greater with genotype AAG/AAG (c.i. = 1.2–2.7; $P = 0.002$; **Table 5**) and 1.8 times greater with genotype AGG/AGG (c.i. = 1.0–3.3; $P = 0.045$; **Table 5**). The marginal significance of the AGG/AGG genotype is due to the fact that only few individuals carried this genotype. The AGG/AGG genotype is biologically relevant, however, as it was associated with low VEGF levels in both the Belgian and Swedish populations (**Fig. 1b,f**). The association of the at-risk genotypes with ALS is specific, as individuals who were homo- or heterozygous with respect to other haplotypes had no significantly greater risk for ALS, nor were their VEGF blood levels significantly lower.

When assessing the total risk associated with *VEGF* variations (AAG/AAG and AGG/AGG together), we found significant associations for the Swedish individuals with ALS (OR = 1.8; c.i. = 1.2–2.7; $P = 0.006$) as well as the individuals with SALS from Belgium (OR = 1.8; $P = 0.018$) and Birmingham (OR = 3.0; $P = 0.011$), but there was no association for the individuals with SALS from London (OR = 1.1; $P = 0.44$). To determine the net increase in the total number of individuals with ALS resulting from the influence of the *VEGF* at-risk genotypes in many individuals, we calculated the population attributable risk for genotypes AAG/AAG and AGG/AGG together. This value was 5.7% in the Swedish individuals with ALS (relative risk (RR) = 1.7; frequency (f) = 0.094) 5.6% in the Belgian individuals with SALS (RR = 1.7; f = 0.085) and 10.4% in the

Table 5 Distribution of haplotypes defined by the -2,578, -1,154 and -634 SNPs

Haplotype	Sweden			Belgium			Birmingham			London			Pooled
	Controls (n = 554)	SALS (n = 292)	OR (c.i.), P value	Controls (n = 426)	SALS (n = 153)	OR (c.i.), P value	Controls (n = 96)	SALS (n = 90)	OR (c.i.), P value	Controls (n = 143)	SALS (n = 158)	OR (c.i.), P value	OR (c.i.), P value
CGG/CGG	0.05	0.04	0.8 (0.4–1.6)	0.02	0.03	1.4 (0.5–4.2)	0.02	0.03	1.6 (0.3–9.9)	0.05	0.04	0.8 (0.2–2.2)	0.8 (0.6–1.5), 0.94
CGG/CGC	0.16	0.11	0.7 (0.4–1.1)	0.12	0.11	0.9 (0.5–1.6)	0.07	0.12	1.8 (0.7–4.8)	0.14	0.18	1.3 (0.6–2.5)	0.9 (0.7–1.2), 0.47
CGG/AGG	0.10	0.09	0.9 (0.6–1.5)	0.05	0.06	1.1 (0.5–2.4)	0.08	0.08	0.9 (0.3–2.7)	0.07	0.07	1.0 (0.4–2.4)	1.0 (0.7–1.4), 0.90
CGG/AAG	0.14	0.13	0.9 (0.6–1.4)	0.11	0.11	1.1 (0.6–1.9)	0.07	0.11	1.6 (0.6–4.4)	0.13	0.10	0.8 (0.4–1.6)	1.0 (0.7–1.3), 0.91
CGC/AGG	0.11	0.10	0.9 (0.5–1.4)	0.12	0.12	1.0 (0.6–1.7)	0.14	0.09	0.6 (0.2–1.6)	0.07	0.08	1.1 (0.5–2.6)	0.9 (0.7–1.2), 0.49
CGC/AAG	0.15	0.19	1.3 (0.9–1.9)	0.23	0.23	1.0 (0.7–1.6)	0.32	0.18	0.5 (0.2–0.9)	0.19	0.18	0.9 (0.5–1.7)	1.0 (0.8–1.3), 0.99
CGC/CGC	0.07	0.08	1.2 (0.7–2.0)	0.15	0.08	0.5 (0.3–1.0)	0.11	0.08	0.7 (0.3–2.0)	0.11	0.09	0.8 (0.4–1.8)	0.8 (0.6–1.1), 0.19
AAG/AGG	0.10	0.08	0.8 (0.5–1.3)	0.11	0.12	1.1 (0.6–2.0)	0.11	0.11	1.1 (0.4–2.7)	0.09	0.13	1.6 (0.7–3.4)	1.0 (0.7–1.4), 0.92
AGG/AGG	0.03	0.04	1.3 (0.6–2.9), 0.49	0.01	0.05	5.1, 0.006*	0.01	0	–	0.02	0.04	1.8, 0.20*	1.8 (1.0–3.3), 0.045
AAG/AAG	0.07	0.12	2.0 (1.2–3.2), 0.007	0.08	0.10	1.3, 0.19*	0.06	0.19	3.5, 0.006*	0.11	0.10	0.9, 0.5*	1.6 (1.2–2.3), 0.002

*The one-tailed P value calculated in the direction of the AAG/AAG and AGG/AGG at-risk genotypes. In the individual populations, P values are only shown for AGG/AGG and AAG/AAG genotypes, as these were significantly associated with ALS in the single overall logistic regression.

Results for rare genotypes are not shown. The AGG/AGG genotype was not detected in the British SALS population.

Birmingham individuals with SALS (RR = 2.6; $f = 0.073$). We found that the association was most significant when all populations were pooled, with a 1.7 times greater risk for ALS (c.i. = 1.3–2.2; $P = 0.0002$ after adjusting for the country of each population). Notably, we obtained an even more statistically significant association (OR = 1.8; $P = 0.00004$) by calculating the Mantel–Haenszel common odds ratio (Breslow–Day homogeneity test, $P = 0.32$; **Fig. 1g**). Logistic regression analysis setting genotypes AAG/AAG and AGG/AGG as the dependent variable, after adjusting for the country of origin, indicated that these at-risk genotypes conferred a risk for ALS in the pooled populations, independently of the sub-type of disease (typical ALS or progressive bulbar paresis), age, gender or survival after diagnosis (data not shown).

Low levels of Vegfa and ALS in *SOD1^{G93A}* mice

The genetic association and gene expression results indicate that low VEGF-producing alleles are associated with greater risk for ALS. But, for this association to be relevant in the pathogenesis of ALS, VEGF should also have a biological effect on motoneurons. Our previous work in *Vegfa^{Δ/Δ}* mice suggested such a functional involvement¹⁷, and here we sought additional evidence that Vegfa stimulated motoneuron survival in two stress scenarios. First, we analyzed whether low levels of Vegfa modified motoneuron degeneration in an established ALS mouse model, and second, we evaluated whether delivery of Vegfa could prevent motoneuron death in ischemic conditions, as ischemia might be a mechanism of motoneuron degeneration¹⁷. To confirm that reduced Vegfa expression could modify the severity of motoneuron degeneration, we intercrossed mice expressing a hemizygous *SOD1^{G93A}* transgene (an established mouse model of ALS²⁵) with *Vegfa^{Δ/Δ}* mice expressing reduced levels of Vegfa owing to a targeted deletion of the HRE¹⁷. We mated the resultant *SOD1^{G93A} Vegfa^{Δ/+}* mice to obtain *SOD1^{G93A} Vegfa^{Δ/Δ}* and *SOD1^{G93A} Vegfa^{+/+}* littermates with matched genetic backgrounds. *SOD1^{G93A}* mice developed severe muscle weakness due to motoneuron loss beyond 3 months of age, were able to stay for 2 min on a rotarod until 118 ± 4 d of age and died after 124 ± 4 d, whereas *Vegfa^{Δ/Δ}* mice developed the first signs of muscle weakness and motoneuron loss after 5 months of age and survived for more than a year¹⁷. Compared with *SOD1^{G93A} Vegfa^{+/+}* littermates, *SOD1^{G93A} Vegfa^{Δ/Δ}* mice had significantly more muscle weakness, performed significantly worse in the rotarod test (they were unable to stay for 2 min on the rotarod beyond 98 ± 2 d; $n = 8$; $P = 0.0001$) and died earlier (107 ± 3 d; $n = 10$; $P = 0.001$; **Fig. 2a**). A more complete description of the

SOD1^{G93A} Vegfa^{Δ/Δ} phenotype will be reported elsewhere. These findings indicate that a promoter mutation, which reduces Vegfa expression in mice, is capable of modifying the motoneuron degeneration phenotype in an ALS mouse model.

Paralysis after spinal ischemia in *Vegfa^{Δ/Δ}* mice

If VEGF functionally affects motoneurons, levels of VEGF might be expected to regulate motoneuron survival in conditions of stress. Because ischemia might contribute to motoneuron degeneration¹⁷, we challenged *Vegfa^{Δ/Δ}* mice (having lower Vegfa levels) with spinal cord ischemia reperfusion at 7 weeks of age, when their motor function was

Figure 2 Role and therapeutic potential of VEGF in stimulating motoneuron survival. (a) Kaplan–Meier survival curve showing the shorter survival of *SOD1^{G93A} Vegfa^{Δ/Δ}* mice relative to *SOD1^{G93A} Vegfa^{+/+}* littermates ($P = 0.001$). (b,c) Illustration of the neurological outcome after acute spinal cord ischemia. (b) Mouse with score 1: hind limbs stretched and rigid when lifted by tail (upper panel), normal walking (lower panel). (c) Mouse with score 4: hind limbs immobile when lifted by tail (upper panel), unable to walk (lower panel). (d) Minimal spinal cord ischemia, which caused only transient motor impairment in wild-type mice, caused persistent paralysis in *Vegfa^{Δ/Δ}* mice. $*P < 0.05$. (e) Administration of VEGF, but not PGF, to wild-type mice improves motor recovery after severe spinal cord ischemia. $*P < 0.05$. (f–k) Immunostaining of motoneurons in the anterior horn at the lumbar level for NeuN (f–h) or SMI32 (i–k) from control mice (f,i) or mice after spinal cord ischemia (g,h,j,k). There were more immunoreactive neurons (arrowheads) after delivery of VEGF (h,k) compared with saline (g,j). SMI32 stains non-phosphorylated neurofilaments, which are normally detected in the soma (gray matter) but become detectable in the axons (white matter) on cellular stress. The border between gray and white matter is delineated with an interrupted line.

still normal and no disease symptoms were yet observed¹⁷. We scored motor function in the hind limbs 0, 6, 24, 48 and 72 h after reperfusion using a rating scale of 0 (normal function) to 5 (absence of movement; Fig. 2b,c). To assess whether *Vegfa*^{Δ/Δ} mice might be more sensitive to ischemia reperfusion, we clamped the aortic arch, left subclavian artery and internal mammary artery for only 8 min, as this insult caused only transient paralysis in wild-type mice (Fig. 2b,d). Only one of six wild-type mice had a neurological score of 1 after 3 d. In contrast, all six *Vegfa*^{Δ/Δ} mice were severely paralyzed for the entire duration of the analysis (3 d) and they did not recover neurologically ($n = 6$; $P = 0.0004$; Fig. 2c,d). Thus, a relatively minor ischemic insult that caused only a transient clinical deficit in wild-type mice caused persistent paralysis in *Vegfa*^{Δ/Δ} mice because of their reduced levels of Vegfa.

Administration of VEGF protects mice against motoneuron loss

Encouraged by the genetic evidence in humans and mice that Vegfa levels functionally determine motoneuron survival, we analyzed whether delivery of VEGF to wild-type mice might salvage motoneurons at risk of irreversible damage after spinal cord ischemia reperfusion, a model previously used to evaluate the therapeutic potential of riluzole, a drug currently used to treat ALS^{26,27}. Because clamping the thoracic vessels for only 8 min resulted in a transient paralysis with almost complete recovery after 24 h, we developed a more severe model of motoneuron loss to evaluate the therapeutic potential of VEGF. When we prolonged the clamping duration to 12 min, all saline-treated wild-type mice were severely paralyzed and only partially recovered after 3 d (Fig. 2e). Daily intraperitoneal administration of 2 μg VEGF significantly protected wild-type mice against permanent paralysis. Despite a similar degree of paralysis immediately after clamping, the neurologic outcome of VEGF-treated mice improved more rapidly and extensively ($n = 12$; $P = 0.0004$; Fig. 2e). The effect of VEGF was specific, as delivery of the VEGF homolog PGF (previously called PLGF), which binds a different receptor and does not affect motoneuron survival¹⁷, was ineffective ($n = 5$; $P =$ not significant; Fig. 2e). Histopathology showed that there was severe loss of motoneurons in the lumbar gray matter of saline-treated mice but milder injury in VEGF-treated mice. Of the 126 ± 11 NeuN-positive neurons normally present per ventral horn section in unligated mice, $47 \pm 5\%$ were lost after spinal cord ischemia in saline-treated mice, but only $32 \pm 3\%$ were lost in VEGF-treated mice ($n = 9$; $P < 0.05$; Fig. 2f–h). Staining of non-phosphorylated neurofilaments using the SMI32 antibodies showed an even more significant protection by VEGF; of the 23 ± 2 SMI32-positive neurons normally present per ventral horn section in unligated mice, only $20 \pm 3\%$ remained after spinal cord ischemia in saline-treated mice, but up to $37 \pm 4\%$ were still present in VEGF-treated mice ($n = 9$; $P = 0.01$; Fig. 2i–k). NeuN- and SMI32-positive neurons in the ventral horn at the thoracic level were comparable in all groups of mice (data not shown). Thus, whereas reduced VEGF levels predispose to motoneuron loss in mice and humans, delivery of VEGF protects against loss of at-risk motoneurons after stress.

DISCUSSION

The pathogenesis of SALS is largely unknown^{1–4,7,10,15}. Even though VEGF does not map to previously identified genomic regions linked to ALS^{6,7}, the genetic findings in *Vegfa*^{Δ/Δ} mice, in which we modified the HRE in the VEGF promoter¹⁷, raised the question of whether VEGF might act in the genetic pathway of motoneuron degeneration in humans. Because we detected no sequence variations in the HRE sequence of over 400 individuals with ALS from different European countries, we examined whether

variations of SNPs in VEGF might confer greater risk for ALS. In particular, we focused on the SNPs –2,578C→A, –1,154G→A and –634G→C, as they were previously documented to correlate with downregulated VEGF expression^{19,20}. Genotyping populations of affected individuals and controls from Sweden, Belgium and England showed that these individual SNPs had strong linkage disequilibrium and, as is often the case, interacted with each other to determine, in combination, susceptibility to ALS. Of all possible combinations, haplotypes –2,578A/–1,154A/–634G (AAG) and –2,578A/–1,154G/–634G (AGG) were significantly associated with greater risk for ALS. Even though the risk associated with the at-risk genotypes AAG/AAG and AGG/AGG differed in the individual populations, the overall risk for ALS was consistently 1.8–3.0 times greater when analyzing both at-risk genotypes together. Only in the greater London population did we not find an association. When assessing the risk in a meta-analysis of all populations and after adjusting for the population of origin, however, genotypes AAG/AAG and AGG/AGG together had a 1.8 times greater risk ($P = 0.00004$). Calculation of the population-attributable risk indicated that the influence of the VEGF at-risk genotypes resulted in a net increase in the total number of individuals with ALS by 5.7, 5.6 and 10.4% in the Swedish, Belgian and Birmingham populations, respectively. Although this may seem a modest effect, it is relevant when comparing it to the role of mutations in *SOD1*, which are responsible for 1–2% of the entire ALS population^{1–4,7,10,15}. Defining the attributable risk of VEGF genotypes in ALS subpopulations might provide further insights into pathogenesis and therapeutic interventions.

Only 5–10% of all individuals with ALS have a family history and of those only 14% (France) to 23% (US and Scandinavia) are attributable to mutations in *SOD1* (refs. 6,7). It is thus noteworthy that we found a similar association of VEGF genotypes in individuals with FALS and with SALS who have no mutations in *SOD1*, as the pathogenesis of FALS is probably heterogeneous. It indicates that VEGF has a relevant role in modifying motoneuron degeneration, even when inherited disease genes determine the latter. Because of the limited availability of individuals with FALS with mutations in *SOD1*, we could not analyze whether variations in VEGF would also modify the disease characteristics in individuals with FALS carrying mutations in *SOD1*. But mice carrying mutations in both *SOD1* (ref. 25) and the *Vegfa* promoter (*SOD1*^{G93A} *Vegfa*^{Δ/Δ} mice) died earlier because of more severe motoneuron degeneration. These genetic data in mice thus underscore the need for evaluating whether VEGF also modifies motoneuron degeneration in individuals with FALS and mutations in *SOD1*.

Although each of the at-risk alleles that we identified has previously been correlated with lower VEGF levels *in vivo* or *in vitro*^{19,20,28}, we cannot exclude the possibility that they are in linkage disequilibrium with additional (perhaps more functionally relevant) SNPs. We found, however, that VEGF plasma levels were most significantly reduced in individuals with ALS and in healthy individuals with the at-risk genotypes AAG/AAG or AGG/AGG and that these at-risk genotypes reduced expression of VEGF *in vitro*. Moreover, our findings indicate that the at-risk –1,154A allele reduces VEGF gene transcription, and the –634G allele impairs IRES-dependent translation of the normally secreted VEGF isoforms, thus suggesting a molecular explanation for the previously documented lower expression of VEGF by cells or subjects carrying these alleles^{19,20,28}. The unusually long 5' UTR of VEGF contains two internal ribosome entry sites (IRES-A at nucleotide –294 to –1 and IRES-B at nucleotide –948 to –556) involved in the enhancement of translation

of AUG-initiated VEGF²¹. Analysis of the predicted secondary structure of the 5' UTR indicated that the secondary structure of the IRES-B sequence was significantly remodeled for the common -634G or variant -634C allele. Because an optimal secondary structure is essential for normal IRES function, a possible explanation for the impaired IRES-dependent VEGF translation by the -634G allele is that the IRES-B secondary structure of this allele was less optimal than that of the -634C allele. Precedent for diseases caused by mutation of the IRES function is exemplified by a mutation in the nerve-specific connexin 32 causing the Charcot-Marie-Tooth disease²⁹. An interesting question emerging from these data is whether the haplotypes are important for greater risk of ALS or whether this effect is instead determined by the individual alleles. Although the SNP genotypes themselves were sufficient to obtain a statistically significant association in the meta-analysis, we found a more consistent and significant association at the haplotype level. Another unexplained question is why the risk of ALS was not greater in heterozygous AAG/AGG individuals and why their reduced circulating VEGF levels were not as low as those of the homozygous at-risk individuals.

We observed that the -634G allele impaired production of the L-VEGF isoform. L-VEGF is 205 amino acid residues longer than the AUG-initiated VEGF forms because translation is initiated at CUG1 (nt -540 in the 5' UTR; ref. 22). Analysis of the secondary structure of the 5' UTR showed that this initiation codon is located in the immediate vicinity of -634G→C and could therefore be influenced by variations of this polymorphic site. L-VEGF is proteolytically cleaved at the peptide signal sequence and converted to the shorter isoforms (VEGF¹²¹, VEGF¹⁶⁵ and VEGF¹⁸⁹ isoforms; Fig. 1a; ref. 22), which are secreted into the extracellular milieu and known to have biological effects. Although the role of L-VEGF is not clear, it may serve as a reservoir for generation of the shorter isoforms²¹. It is therefore tempting to speculate that insufficient production of the L-VEGF by the -634G allele might have contributed to the reduced VEGF expression levels, which we identified in the present study as a risk factor of ALS. If true, this would provide the first insight in a pathophysiological role of the L-VEGF isoform in disease. Support for such a hypothesis is provided by previous evidence that N-terminally extended isoforms of bFGF or c-myc, the translation of which is also initiated in the 5' UTR, have been implicated in medically important disorders^{30–32}.

We could not document any sequence variation in the HRE of the VEGF promoter. Because HIF1A and EPAS1 bind the HRE, mutation in these genes could also affect expression of VEGF. But we did not detect any functionally relevant missense mutations in *HIF1A* and *EPAS1*. How can we then reconcile the mouse and human genetic data? Notably, we previously documented that baseline VEGF expression levels in *Vegfa*^{Δ/Δ} mice were reduced by ~25%, presumably because the HRE deletion also interfered with additional transcriptional regulators¹⁷. We therefore speculate that variant alleles resulting in reduced VEGF expression levels in baseline normoxic conditions as well as in response to hypoxia might lead to motoneuron degeneration. The lack of functional variations in the HRE or in *HIF1A* and *EPAS1* does not exclude the possibility that impaired hypoxic regulation of VEGF might still contribute to greater ALS risk. In fact, the hypoxia-induced increase in IRES-B activity of the -634C but not of the -634G allele supports such a hypothesis. Considering the complexity of the transcriptional and translational regulation of VEGF expression³³, additional SNPs might also be involved in determining the susceptibility to ALS.

Genetic association studies are often plagued by irreproducibility, particularly when a single, small population (<150 subjects) is

studied^{24,34}. We therefore did meta-analysis on a large population and replicated the association in geographically different populations. Our analysis in almost 2,000 individuals is, to the best of our knowledge, the largest genetic association study with ALS, a disease that is less common than complex traits, such as diabetes or hypertension. After analyzing four different populations, we found a significant association of the low VEGF-producing haplotypes with ALS in 1,668 individuals from three geographically distinct regions (Swedish, Belgian, British/Birmingham) but no association in the 301 individuals from the British/London study group. Such inconsistency in replication is a well recognized phenomenon in genetic association studies^{24,34}. Although we do not know the precise reason for the lack of association in the London study, it does not necessarily negate the positive association in the other populations or minimize the relevance of the overall association on meta-analysis of the pooled populations³⁵. It is also worth noting that VEGF is not an obligatory disease-associated gene like *SOD1*, mutations in which cause ALS regardless of the genetic background or environmental influences. Instead, VEGF is a modifier of ALS whose influence may depend on genetic background, additional modifiers, environment or lifestyle. It is well known that VEGF levels are influenced by environmental changes. Future analysis of how VEGF genetically interacts with previously proposed modifier genes, such as *PSEN1* (ref. 13), *SOD2* (ref. 36), *CNTF*³⁷, *APOE*¹⁶, *LIF*³⁸, or *SMN2* (ref. 14), in determining the overall disease susceptibility might also be helpful.

The genetic association of low VEGF-producing haplotypes with ALS in humans was not the result of a random genome-wide scan but instead was prompted by and now replicates our previous mouse genetic findings that low *Vegfa* levels contribute to greater susceptibility to motoneuron death¹⁷. Our findings that *Vegfa*^{Δ/Δ} mice are more sensitive to a relatively minor ischemic insult, which causes only a transient clinical deficit in normal mice, underscores the conclusion that VEGF levels determine the susceptibility to motoneuron disease. Moreover, the more severe motoneuron death in *SOD1*^{G93A} *Vegfa*^{Δ/Δ} mice, the protective effect of VEGF against ischemic motoneuron death and the low plasma levels of VEGF in individuals with ALS relative to spouses all support a functional role of VEGF, as suggested by the human genetic association. A function for VEGF in neuronal survival, regeneration, growth, differentiation and axonal outgrowth is also implied by a growing body of recent publications^{39–47}. Although our VEGF treatment data only relate to acute spinal cord ischemia, our findings may bear some relevance to ALS, as chronic ischemia might contribute to motoneuron death¹⁷. The findings also raise the intriguing question whether more long-term treatment with VEGF might delay the onset or slow the progression of adult-onset motoneuron degeneration as well.

METHODS

Study populations and sample collection. We analyzed populations from Sweden, Belgium and England (Table 1). None of the individuals with SALS had a documented familial history of ALS to the first degree of relatedness.

Swedish population. Individuals with ALS were unrelated citizens who reported (northern) Swedish citizenship for at least three generations and were referred to the Umea University ALS Clinic. Only individuals with SALS or FALS without mutations in *SOD1* were included. For the Swedish control samples, we used 275 individuals from the Betula prospective cohort study of Umea, Sweden⁴⁸. To match the Betula controls to the individuals with ALS as a group, we sampled controls from age cohorts of 50, 55, 60, 65 and 70 years. Potential control subjects with neurodegenerative disorders were excluded. In all instances, we contacted spouses of individuals with ALS, and a total of 106

spouses agreed to take part in the study. Individuals who immigrated to Sweden during any of the last three generations and their descendants were excluded. We applied similar criteria to select neurological control samples from individuals admitted to the Department of Neurology, Umea University Hospital for non-ALS related neurological disorders (headache, conversion syndrome, epilepsy, hydrocephalus, stroke, multiple sclerosis, multifocal motor neuropathy, etc.).

Belgian population. The Belgian population of individuals with ALS consisted of 153 unrelated subjects of Flemish origin who were referred to the University Hospital of Leuven for SALS⁴⁹. The Flemish control population consisted of healthy spouses of individuals with dementia and randomly sampled healthy individuals. The control population was matched as a group to have a similar age, geographical and ethnical distribution as the affected individuals. All individuals in the Belgian sample self-reported Flemish ethnicity for at least three generations.

British (Birmingham and London) population. Individuals with SALS were British citizens with British surnames, admitted to the Department of Neurology, School of Medicine in Birmingham or to King's College in London. No details were available about the ancestry of the previous generations, but the included subjects were probably of English, Welsh, Scottish and Irish ancestry. Healthy controls were matched as a group to have similar age, ethnicity and geographic distribution. The Birmingham controls consisted of spouses of individuals with ALS and, if unavailable, spouses of individuals with Parkinson disease. The London controls were healthy controls sampled from the hospital staff.

Clinical data. We obtained the following clinical data from affected individuals: gender, age at onset of disease and duration of disease (or survival; categorized into three classes: <4 years, 4–10 years and >10 years for the Swedish population; in months for the Belgian and British populations). We categorized the type of disease at onset as (i) typical ALS with onset in the legs, arms, chest or abdominal muscles and clinical evidence for lesions to both the upper motoneuron systems in the brain and the lower motoneuron systems in the spinal cord or (ii) progressive bulbar palsy, also called ALS with bulbar onset, a type of motoneuron degeneration with onset in the muscles in the face, mouth and throat. We analyzed the clinical data blindly before the results of the genetic studies were available. Ethical approval for this study was granted by the Ethics Committee from each of the participating institutions and informed consent was obtained from all subjects.

DNA extraction. We extracted DNA from white blood cells from peripheral blood using the QIAamp Blood Kit (Qiagen).

DHPLC analysis. We screened 250 Swedish and 96 Belgian individuals for SNPs in the HRE by denaturing high-performance liquid chromatography (DHPLC) analysis, using a Transgenomic WAVE instrument. We also screened 96 Swedish individuals with SALS and 200 controls for variations in the exons and exon/intron boundaries of *HIF1A* and *EPAS1*. We designed primers using the Primer3 software program. We loaded crude PCR products on a DNasep column and eluted them from the column using an acetonitrile gradient in a 0.1 M triethylamine acetate buffer, pH 7, at a constant flow rate of 0.9 ml min⁻¹. The gradient was created by mixing eluent A (0.1 M triethylamine acetate, 0.1 M Na₄EDTA) and eluent B (25% acetonitrile in 0.1 M triethylamine acetate). The gradient and temperature required for successful resolution of heteroduplex molecules were predicted by Wavemaker version 3.4.4. Primer sequences and PCR and sequencing conditions are available on request.

DNA sequencing. We used direct sequencing (Big Dye terminator cycle sequencing kit, Applied Biosystems) of PCR fragments with aberrant DHPLC patterns to confirm the presence of a variant allele.

Pyrosequencing. We genotyped SNPs in the promoter and 5' UTR by pyrosequencing. We immobilized biotinylated PCR products on streptavidin-coated paramagnetic beads (Dynal). We obtained biotinylated single-stranded DNA by incubating the immobilized PCR products in 50 µl 0.5 M NaOH for 5 min

and then washing twice in 100 µl 10 mM Tris-Acetate, pH 7.6. We carried out primer annealing by incubation at 80 °C for 2 min and then at room temperature for 5 min. We carried out pyrosequencing on the PSQ96 pyrosequencer (Pyrosequencing). To assess population substructure, we pyrosequenced five biallelic SNPs (on chromosome 2, 5, 7, 17 and 18) for the four control populations. In addition, we genotyped a panel of five tetranucleotide STR markers (5, 10, 12, 15, 21). Primer sequences and PCR and sequencing conditions are available on request.

VEGF levels. To determine plasma VEGF levels, we collected blood in 10-ml vacuum tubes containing EDTA (Becton Dickinson Vacutainer Systems Europe), quickly centrifuged it and immediately stored plasma fractions at –80 °C until analysis. To determine VEGF concentrations in serum, we drew blood into serum tubes containing clot activator (Becton Dickinson Vacutainer Systems Europe) and allowed it to coagulate for at least 30 min at room temperature before centrifugation. We subdivided samples into aliquots and stored them at –30 °C until analysis. We drew samples during morning office hours. We used the commercial Quantikine human VEGF ELISA kit (R&D System) to measure plasma and serum concentrations of VEGF.

Statistical analysis. Common or variant alleles were defined as the allele with the highest or lowest frequency, respectively. Haplotypes were considered rare when their frequency was 1% or less. Deviations from Hardy–Weinberg equilibrium were tested using the observed allele frequency by the χ^2 contingency test (1 degree of freedom, d.f.). We assessed differences in distribution of alleles and genotypes between the groups by calculating the Pearson χ^2 , from which we derived *P* values with the appropriate d.f. number (2 d.f. for genotypes, 1 d.f. for alleles or genotypes under a recessive model). As the descriptive measure of association between genotypes and alleles, we calculated crude ORs, their 95% c.i. ranges and corresponding *P* values relative to the common (for the –1,154G→A and –2,578C→A SNP) or variant (for –634G→C SNP) genotype or allele. To adjust ORs for age and gender, we carried out logistic regression using the statistical software program SPSS version 10.0, yielding similar levels of association and statistical significance. We used the maximum-likelihood method to estimate haplotype frequencies (EH program⁵⁰) to determine estimates of disequilibrium (D_{ij}) between pairwise combinations of alleles (here $D_{ij} = h_{ij} - p_i q_j$ and p_i and q_j are the frequencies for allele *i* at locus 1 and for allele *j* at locus 2). We calculated the linkage disequilibrium coefficient D'_{ij} standardized by its maximum value as $D' = D/D_{\max}$. If $D < 0$, then $D_{\max} = -pq$; if $D > 0$, then $D_{\max} = p(1 - q)$. We analyzed differences in frequencies of particular genotypes (hetero- or homozygosity with respect to haplotypes) between affected individuals and controls by calculating logistic odds ratios of the at-risk genotypes relative to the other genotypes as a group. When analyzing pooled populations, we pooled individual genotypes of affected individuals and controls and carried out logistic regression to adjust for the population of origin. We tested the specific hypothesis, suggested by our previously published transgenic mouse data¹⁷, that reduced VEGF levels might increase the risk for motoneuron degeneration by genotyping in the Swedish population those SNPs in the promoter and 5' UTR of *VEGF* predicted to reduce *VEGF* expression^{19,20}. Then, to replicate our findings, we compared individuals with SALS in two other populations (Belgian and British) with their randomly ascertained local controls. For the Swedish population and all pooled analyses, we calculated two-tailed *P* values. In replication populations (British and Belgian), we calculated one-tailed *P* values for all alleles in the direction of their appearance in the at-risk haplotype AAG. Because we knew in advance the direction of the test in the replication studies and wanted to test only the influence of the at-risk genotypes, we used one-tailed probabilities. We obtained one-sided type I probabilities for the χ^2 test by halving the two-sided probabilities calculated by SPSS. For ORs, there were no confidence intervals when one-tailed *P* values were calculated. Results were considered statistically significant when their type I probability was less than 0.05. We did not carry out a Bonferroni correction for several reasons: (i) we tested a specific hypothesis that was suggested by previously obtained biological data¹⁷; (ii) this hypothesis was sustained by the replication studies and extended by the correlation of VEGF levels with the at-risk genotypes and haplotypes; (iii) the three SNPs did not segregate independently but were in significant linkage disequilibrium; and (iv) we first tested the distribution of all genotypes (hetero- and homozygosity with respect to haplotypes)

together in a single overall logistic regression, accounting simultaneously for the country of origin. We evaluated the at-risk genotypes in the separate subpopulations only because this logistic regression was overall significant ($P = 0.046$) and because we found significant ORs for genotypes AAG/AAG and AGG/AGG (on their own relative to all other genotypes). To calculate the Mantel-Haenszel common OR of the at-risk genotypes and test for homogeneity (Breslow-Day homogeneity test) we used SPSS. The population attributable risk was estimated using the Levin formula: $PAR = 100 \times (\text{proportion of total population exposed} \times (\text{relative risk for exposed versus nonexposed} - 1)) / ((\text{proportion of total population exposed} \times (\text{relative risk for exposed versus nonexposed} - 1)) + 1)$. We calculated relative risk ratios as the ratio of at-risk frequencies between affected individuals and controls. We evaluated the distribution of the quantitative variables by the Kolmogorov-Smirnov test for Gaussian normality. When the distribution was Gaussian, we compared quantitative variables between groups by parametric analysis of variance (e.g., expression experiments). When this was not the case, such as for the VEGF levels, we used the non-parametric Kruskal-Wallis analysis of variance to compare several groups and the Mann-Whitney U test to compare their central tendency measures. We used SPSS version 10 for these tests. To assess the degree of population substructure in the four control sample sets, we selected 96 Swedish (population-based), 96 Belgian and 48 Birmingham spouses and 48 London control samples and genotyped them for five SNPs and five STRs from five different chromosomes and with an allele frequency of >0.25 in each population. Hardy-Weinberg equilibrium was fulfilled for all SNPs and STRs in each population, except for one SNP in the Belgian sample group ($P = 0.024$), suggesting that large-scale population differences are not apparent in the samples.

Luciferase reporter assays. To test the relative ability of the various haplotypes (e.g., AAG, AGG and CGC) to modify gene expression, we fused the fragment from $-2,469$ to $-2,714$ (harboring the $-2,578C \rightarrow A$ SNP) to the fragment from $-1,176$ to -405 (harboring the $-1,154G \rightarrow A$ and $-634G \rightarrow C$ SNPs) and cloned it into the firefly luciferase plasmid pGL3-Basic (Promega). We introduced the various $-2,578$, $-1,154$ or -634 alleles using the site-directed mutagenesis kit (Stratagene). We transfected reporter plasmids into human glioma GI-1 cells (RCB0763, RIKEN Cell Bank), grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum, using the FuGENE6 Transfection Reagent (Roche Diagnostics). As an internal control, we cotransfected $0.1 \mu\text{g}$ of the renilla luciferase plasmid pRL-TK containing the herpes simplex virus-thymidine kinase (HSV-TK) promoter with $1 \mu\text{g}$ of the firefly luciferase vector. At 16 h after transfection, we cultured cells for 8 h in normoxia or hypoxia (2% O_2) and collected them for measurement of firefly and renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega). We also evaluated the transcriptional activity of the $-2,578C \rightarrow A$ SNP alone by cloning the fragment from $-2,469$ to $-2,714$ in front of the minimal CMV promoter, driving the luciferase reporter gene, in the pGL3 promoter plasmid. We similarly evaluated the transcriptional/promoter activity of the isolated $-1,154G \rightarrow A$ SNP by cloning the fragment from $-1,172$ to -987 in front of the luciferase marker gene in the pGL3 basic plasmid. We introduced the various $-2,578$ or $-1,154$ alleles using the site-directed mutagenesis kit (Stratagene).

IRES-B activity. We used a bicistronic vector containing two different reporter cistrons, the renilla and firefly genes encoding the luciferase enzyme, designated LUCr and LUCf, respectively, separated by the 5' UTR of VEGF (from -947 to -484) in the intercistronic region²¹. Translation of LUCr is cap-dependent, whereas translation of LUCf is dependent on the IRES-B in the 5' UTR region upstream of LUCf. The LUCf/LUCr ratio after transfection in C6 glioma cells served as a measure of the relative IRES-B activity.

L-VEGF production. We transfected COS cells with an expression cassette containing the entire 5' UTR (including the CUG1 start codon of L-VEGF at -540) in-frame with the cDNA encoding the matrix-attached VEGF¹⁸⁹ isoform²². We cloned a cassette containing the common $-634G$ or variant $-634C$ allele by site-directed mutagenesis. This L-VEGF was predicted to contain 394 amino acid residues and have a molecular weight of 43 kDa. After Western blotting using an antibody specific to L-VEGF²², we used densitometry to quantify the L-VEGF and VEGF¹⁸⁹ bands. We carried out experiments at least four times in triplicate.

Mouse studies. We intercrossed mice expressing a $SOD1^{G93A}$ transgene (obtained from the Jackson Laboratory) with $Vegfa^{\Delta/\Delta}$ mice expressing low levels of Vegfa owing to a targeted deletion of the HRE¹⁷. We then mated the resultant $SOD1^{G93A} Vegfa^{\Delta/+}$ mice to obtain $SOD1^{G93A} Vegfa^{\Delta/\Delta}$ and $SOD1^{G93A} Vegfa^{+/+}$ littermates with matched genetic backgrounds. We scored muscle weakness using the rotarod as described¹⁷. We carried out spinal cord ischemia reperfusion by clamping the aortic arch, left subclavian artery and internal mammary artery as described^{26,27}, except that we used normal wild-type mice with a Swiss genetic background or $Vegfa^{\Delta/\Delta}$ mice with a mixed genetic background¹⁷. In control Swiss mice, the clamping time (12 min; 36°C) was optimized to yield a partially reversible paralysis of the hind limbs after 24–72 h with acceptable perioperative mortality (10–15%), allowing us to evaluate the therapeutic potential of VEGF. In $Vegfa^{\Delta/\Delta}$ mice, the thoracic vessels were only clamped for 8 min to obtain a transient and less severe paralysis. Motor function in the hind limbs was scored 0, 1, 3, 6, 24, 48 and 72 h after reperfusion using a rating scale of 0 (normal function), 1 (hind limbs are stretched but remain rigid when the mouse is lifted by its tail, normal walking), 2 (no stretching of hind limbs when lifted by tail, normal walking), 3 (rigid hind limbs when lifted by tail, ataxic or knuckling walking), 4 (rigid hind limbs when lifted by tail, hind limb movement but unable to walk) and 5 (rigid hind limbs when lifted by tail, total absence of movement). We analyzed neuropathological signs of motoneuron loss histologically as described¹⁷. We administered $2 \mu\text{g}$ of VEGF¹⁶⁵ (R&D Systems) or PGF2 (Reliatech) intraperitoneally 1 d before the surgery and then daily until 2 d after surgery. Neurological and histological outcome was scored independently by two investigators blinded to the treatment type.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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