

# A polymorphism in the *EAAT2* promoter is associated with higher glutamate concentrations and higher frequency of progressing stroke

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**It remains unclear why some individuals are susceptible to excitotoxicity after stroke. A possible explanation is impaired glutamate uptake. We have found a highly prevalent polymorphism in the promoter of the glutamate transporter *EAAT2* gene that abolishes a putative regulatory site for activator protein-2 (AP-2) and creates a new consensus binding site for the repressor transcription factor GC-binding factor 2 (GCF2). The mutant genotype is associated with increased plasma glutamate concentrations and with a higher frequency of early neurological worsening in human stroke. After transfection into astrocytes, the mutant promoter was not activated by AP-2 and was effectively repressed by GCF2, and its activity in the presence of GCF2 was reduced when compared with the AP-2-cotransfected wild-type promoter. We also show that GCF2 is expressed in ischemic rat brain, suggesting that decreased glutamate uptake occurs in individuals carrying the mutation after stroke. These findings may explain individual susceptibility to excitotoxic damage after stroke as well as the failure of glutamate antagonists in those patients without this polymorphism.**

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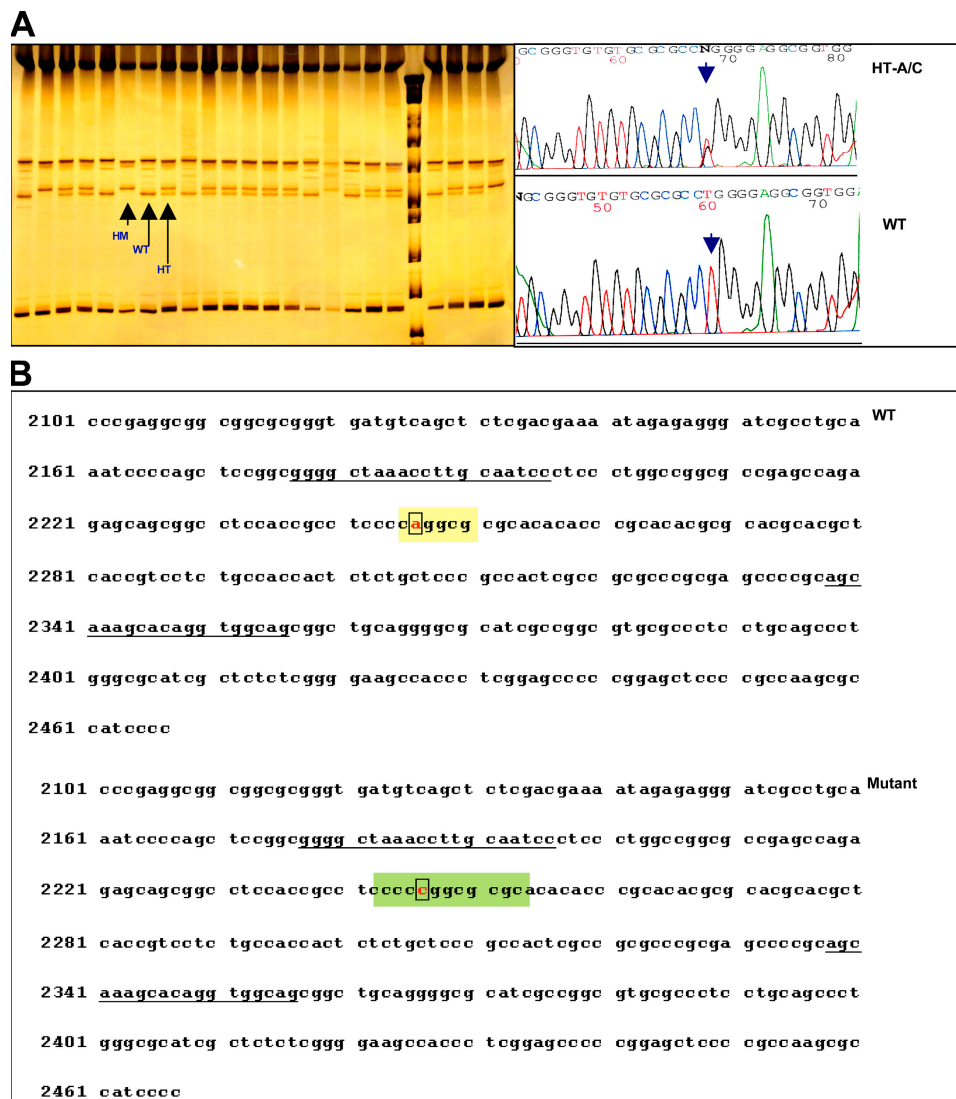
Abbreviations used: AP-2, activator protein-2; CSF, cerebrospinal fluid; GCF2, GC-binding factor 2; MCAO, middle cerebral artery occlusion; SSCP, single-strand conformation polymorphism.

Stroke is the third cause of mortality and the first cause of serious disability in developed countries. Genetic research may be helpful to achieve a better understanding of susceptibility factors related to stroke as well as to determine stroke outcome and even therapeutic responses. Glutamate is the main excitatory neurotransmitter in the mammalian central system. However, excessive levels of extracellular glutamate are excitotoxic and lead to neuronal death (for review see references 1, 2). Cerebral ischemia results in a decrease of oxygen and glucose that is in turn responsible for the release of glutamate at the presynaptic level. The subsequent activation of glutamatergic postsynaptic receptors is the primary process of a cascade of sequential molecular events that finally result in the death of neurons

located both adjacent to and distant from the initial necrotic zone. In humans, the concentrations of glutamate in plasma and cerebrospinal fluid (CSF) have been found to be significantly higher in patients with large cerebral infarcts, and in those with a higher risk of early neurological deterioration, supporting the excitotoxic activity of glutamate in patients with cerebral infarction (3, 4). Interestingly, glutamate levels did not correlate to the initial stroke severity, and their association with progressing stroke was independent of the infarct volume, generating the hypothesis of an individual susceptibility to the glutamate-mediated ischemic injury.

Glutamate transport is the primary and only mechanism for maintaining extracellular glutamate concentrations below excitotoxic levels (for review see references 5–7). This transport is executed by the five high affinity, sodium-dependent plasma membrane glutamate

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**Figure 1. A polymorphism in the promoter region of the EAAT2 gene, consisting of an A-to-C change at –181 bp from the transcriptional start site.** (A) SSCP analysis and complementary sequence of EAAT2 promoter. (B) Bioinformatic approach of wild type and mutant promoters. WT promoter contains a consensus

binding site for AP-2 transcription factor (yellow). The polymorphism abolishes this sequence and creates a new GCF2 consensus binding site (green). The sequence of the primers used in fragment amplification are underlined. WT, wild type; HM, homozygous; HT, heterozygous.

transporter subtypes that have been identified thus far (for review see reference 8). Among them, the astroglial transporter EAAT2 (GLT-1) (9, 10), also found in some neurons, is responsible for up to 90% of all glutamate transport in adult tissue (for review see reference 11). Recently, the human EAAT2 gene promoter has been isolated and characterized, and an elevated expression of the gene has been demonstrated in astrocytes (12). Bioinformatics analysis of the promoter region has revealed several potential regulatory transcription factor-binding elements, including Sp1, NF-κB, N-myc, and NFAT that may contribute to EAAT2 expression and its regulation. Polymorphisms in this region could affect or create novel regulatory transcription factor-binding elements, which could be responsible for differences in both gene func-

tion and regulation under normal and pathological conditions such as cerebral ischemia.

The primary aim of this study was to investigate the presence of functional polymorphisms in the EAAT2 gene that could be associated with increased glutamate concentrations in blood and neurological deterioration in patients with acute ischemic stroke.

**RESULTS**

**Polymorphism analysis**

Molecular analysis of the EAAT2 promoter in the total of 207 individuals (101 patients and 106 controls) allowed the detection of a novel polymorphism. The polymorphism was an A-to-C change at 181 bp from the transcriptional start site

(Fig. 1 A). The prevalence of the polymorphism in stroke patients was comparable to that of the healthy subjects (0.43 vs. 0.39;  $P = 0.4$ ). Sequence analysis of the promoter region revealed that the sequence containing the polymorphism site corresponds to the putative regulatory transcription factor-binding element activator protein-2 (AP-2). The polymorphism abolishes this sequence and creates a new consensus sequence corresponding to the transcription factor-binding element GC-binding factor 2 (GCF2) (Fig. 1 B).

### Genotype-phenotype correlation

No significant differences were found in baseline clinical characteristics between patients with wild and mutant genotype (Table I). Patients with mutant allele C showed higher glutamate concentrations on admission ( $P = 0.024$ ) and at 24 h ( $P = 0.015$ ) (Fig. 2). In agreement with the higher frequency of glutamate levels,  $>200 \mu\text{mol/L}$  in patients with allele C (22.2 vs. 3.4%; Fisher's exact test;  $P = 0.017$ ) early neurological deterioration (defined as an increase  $\geq 4$  points in the National Institutes of Health stroke scale (NIHSS) score between admission and 72 h; reference 13) occurred more frequently in the mutant group than in the wild-type group (23.6 vs. 6.9%; Fisher's exact test;  $P = 0.042$ ) (Fig. 3).

### Activity and regulation of human *EAAT2* promoter transfected into rat astrocytes

Rat astrocytes were transfected with either wild-type or mutant pEAAT2PrLUC (plasmid  $-742/+31$ , MluI-BglII fragment; cloned into the pGL3-basic vector). In these cells, the mutant construct showed a reduction of 30% in promoter activity in comparison with the wild type ( $P < 0.001$ ; Fig. 4, control). After cotransfection with AP-2, wild-type but not mutant pEAAT2PrLUC activity was increased (Fig. 4; 29% increase;  $P = 0.01$ ). Conversely, mutant but not wild-type pEAAT2PrLUC activity was decreased after cotransfection with GCF2 (Fig. 4; 57% reduction with regard to the wild-type promoter activity;  $P < 0.001$ ). Collectively, GCF2-cotransfected mutant showed a reduction in promoter activity of almost 70% when compared with AP-2-cotransfected wild-type (Fig. 4;  $P < 0.001$ ).

### GCF2 expression in normal and ischemic rat brain

The expression of GCF2 was not detected in either cortex or striatum from control rats. However, middle cerebral artery occlusion (MCAO) caused the expression of GCF2, which was detectable 2 h after the occlusion and persisted 24 h after in both cortex and striatum (Fig. 5).

## DISCUSSION

This study has found a novel and highly prevalent polymorphism in the promoter of the *EAAT2* glutamate transporter gene. This polymorphism is not associated with increased risk for stroke because its prevalence is comparable in stroke patients and in healthy subjects. However, this polymorphism was associated with higher and maintained plasma glutamate concentrations as well as with higher frequency of neuro-

logical deterioration in patients with acute hemispheric stroke. Importantly, patients with the mutant genotype had a sixfold increase in the risk of having glutamate levels  $>200 \mu\text{mol/L}$ , a threshold that has been associated with neurological worsening (4). These findings were not explained by differences in the risk factors, baseline characteristics, stroke severity, or diffusion-weighted MRI and perfusion-weighted MRI lesion volumes on admission that were comparable between the two groups.

Our results support the hypothesis that, in patients with acute ischemic stroke, the magnitude of the excitotoxic damage could be genetically determined as the result of a decrease in the glutamate uptake. In this context, it has been reported that plasma and CSF glutamate levels are elevated in patients with acute stroke and that they are threefold higher in patients with subsequent early neurological deterioration than in those with stable or improving stroke (3, 4). In addition, CSF glutamate levels on admission have been found to remain elevated up to 24 h in patients who develop progressing stroke in the following 48 h, whereas they drop to the normal range within the first 6 h after onset of the ischemia in patients without later neurological worsening (14). Because the correlation between CSF and plasma values was high, glutamate levels in blood were attributed to the result of the acute cerebral ischemia. This hypothesis was further demonstrated in an experimental model after MCAO (15).

To explore further our clinical hypothesis, we used rat astrocytes transiently transfected with the mutant *EAAT2* promoter to study whether the polymorphism affects its functional activity. Promoter fragment  $-742/+31$  was chosen based on previous studies where deletion of the most distal region ( $-2426$  to  $-703$ ) did not alter EAAT2-Pr activity (12). The  $-742/+31$  fragments from both wild-type and mutant promoters were cloned into the pGL3-basic luciferase reporter vector and defined as pEAAT2PrLUC-wt or pEAAT2PrLUC-mt, respectively. We have found that the polymorphism identified in this study accounts on its own for a 30% reduction in the basal *EAAT2* promoter activity. The glutamate transporter EAAT2 (GLT-1), together with the glial transporter EAAT1 (GLAST), is responsible for the majority of total glutamate uptake in the brain (for review see reference 8). Partial or total deletion of EAAT2/GLT-1 transporter leads to neuronal degeneration and brain injury (16, 17), whereas optimal functioning of EAAT2/GLT-1 is essential for minimizing neuronal damage after stroke because cerebral ischemia-induced down-regulation of EAAT2/GLT-1 contributes to neuronal death (18, 19). Because a decrease in promoter activity would correlate with decreased expression of EAAT2 transporter, these findings are consistent with greater glutamate concentrations found in patients with the mutant genotype.

To explain how the polymorphism affects the *EAAT2* promoter activity, we performed a bioinformatic approach. A novel finding of our study is that the *EAAT2* promoter region contains a consensus binding site for the transcription factor AP-2. More interestingly, we have found that the

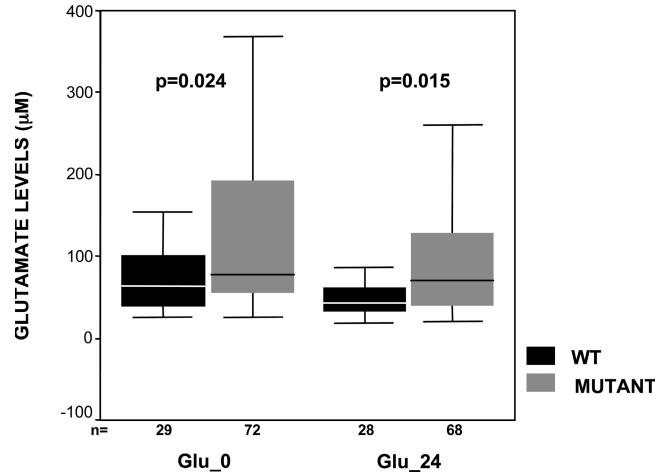
**Table I.** Main characteristics of patients on admission

Characteristic	Wild-type genotype n = 29	Mutant genotype n = 72	p-value
Male, %	48.3	55.6	0.32
Age, yr	66.6 (14.6)	70.3 (8.9)	0.12
Admission delay, min	139.8 (87.6)	175.2 (136.6)	0.19
SBP, mmHg	159.9 (29.6)	167.6 (32.1)	0.26
DBP, mmHg	87.8 (17.5)	88.7 (17.5)	0.81
Serum glucose, mg/dL	127.0 (33.7)	136.1 (55.6)	0.41
Body temperature, °C	36.4 (0.6)	36.4 (0.4)	0.68
Fibrinogen, mg/dL	450.4 (115.0)	444.6 (116.4)	0.82
Early CT signs of infarction, %	73.3	84.8	0.28
Lesion volume, cc			
DWI	19.9 (27.0)	23.6 (36.6)	0.62
PWI <sup>a</sup>	115.1 (129.4)	108.8 (87.1)	0.80
Stroke subtype, %			0.90
Cardioembolic	51.7	52.8	
Atherothrombotic	13.8	16.7	
Undetermined	31.0	29.2	
Other	3.4	1.4	
NIHSS score	13.9 (5.6)	12.2 (6.0)	0.20
Vascular risk factors, %			
Smoking habit	44.8	40.3	0.42
Alcohol intake (>40 mg/d)	20.7	34.7	0.125
Diabetes mellitus	10.3	19.4	0.21
Hypertension	58.6	56.9	0.52
Hyperlipidemia	31.0	31.9	0.56
Previous stroke or TIA	20.7	16.7	0.44
Valvular heart disease	0	6.9	0.17
Atrial fibrillation	32.1	31.4	0.56
Ischemic heart disease	17.2	5.6	0.074

Variables are expressed as mean ± SD or as a percentage, as appropriate. SBP, systolic blood pressure; DBP, diastolic blood pressure; TIA, transient ischemic attack; DWI, diffusion weighted MRI; PWI, perfusion weighted MRI; NIHSS, National Institutes of Health stroke scale.

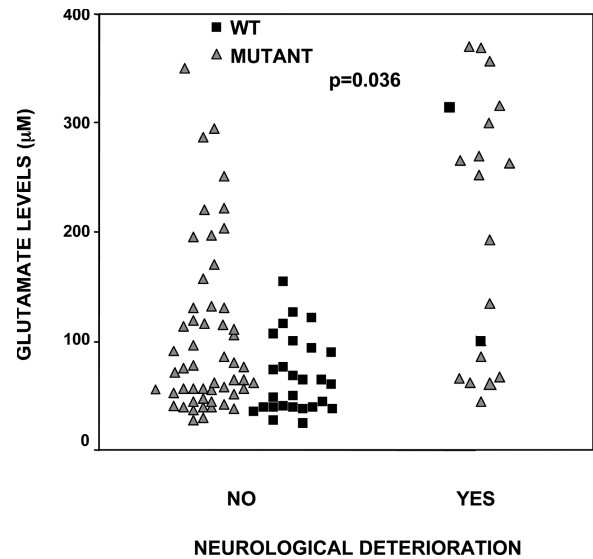
<sup>a</sup>PWI sequences were obtained in 82 patients (23 wild type and 59 mutant genotype). The study was not performed in three patients and the images could not be evaluated in 16 cases due to artefacts related to patients' movement.

polymorphism abolishes this AP-2 consensus sequence and creates a new sequence that corresponds to the consensus binding site for the transcription factor GCF2. AP-2 is a retinoic acid-inducible and developmentally regulated activator of transcription expressed in developing and adult brain (20–22), that mediates transcriptional activation in response to both the phorbol-ester- and diacylglycerol-activated protein kinase C, and the cAMP-dependent protein kinase A pathway (23). Alternatively, GCF2 is a transcriptional repressor that decreases activity of several genes (24). To study the functionality of the polymorphism, the activity of the mutated promoter was studied after cotransfection with these transcriptional regulators. Again, in all groups, pEAAT2PrLUC-mt activity was lower than pEAAT2PrLUC-wt, confirming our previous findings. More interestingly, cotransfection with AP-2 caused a significant increase only in pEAAT2PrLUC-wt activity in agree-

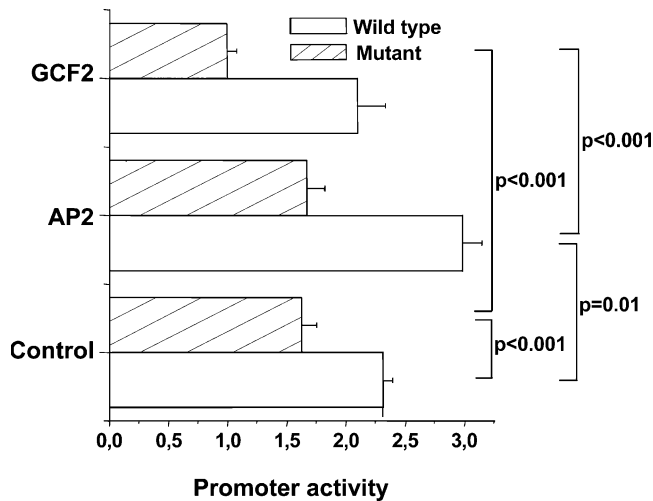


**Figure 2.** Glutamate levels in patients on admission with mutant allele C (mutant) and without (WT). Boxplots show median values (horizontal line inside the box), quartiles (box boundaries), and the largest and smallest observed values (error bars) of baseline (Glu\_0) and 24-h (Glu\_24) plasma glutamate levels in acute stroke patients.

ment with the presence of an AP-2 consensus sequence in the wild-type group, but not in the mutant group. In contrast and accordingly with the new consensus site created by the polymorphism, cotransfection with GCF2 decreased pEAAT2PrLUC activity only in the mutant group. In an attempt to study what would theoretically occur in vivo, we compared the activities of each promoter after cotransfection with the transcription factor whose binding site it contains (i.e., GCF2-cotransfected mutant promoter vs. AP-2-cotransfected wild-type promoter). In these circumstances, there was a drastic reduction (almost 70%) in



**Figure 3.** Glutamate levels on admission by neurological deterioration in patients with wild type (black squares) and mutant genotype (gray triangles). Neurological deterioration in patients with mutant allele C was more frequent and glutamate levels were higher compared with those of the wild-type genotype.

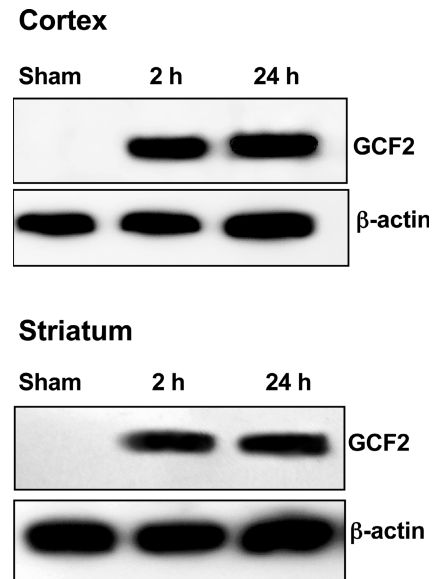


**Figure 4.** Activity of wild-type and mutant *EAAT2* promoter constructions transfected into cultured rat astrocytes, either alone or cotransfected with AP-2 (SPRSV-AP-2) or GCF2 (pGST/GCF2).

the activity of the mutant promoter. These data confirm the following: (a) *EAAT2* promoter activity is increased by AP-2 in the wild-type group; (b) the polymorphism described in our study not only causes the loss of this activating pathway but originates a GCF2-mediated inhibitory one; and (c) this polymorphism remarkably affects the regulation pattern of expression of this glutamate transporter.

Western blot has been performed in normal and ischemic brain to determine whether this regulatory scheme mediated by GCF2 is present in brain, as it would explain and exacerbate the differences observed in patients with mutant and wild-type genotype with ischemic stroke. Although AP-2 expression is known to be expressed in both neurons and astrocytes (25), GCF2 expression in brain appears to be very low (26). Its brain expression after ischemic injury was unknown, but previous evidence suggested it may be induced as is the case in other settings, such as after mechanical injury of cells in culture as well as after balloon injury of rat carotid artery wall (27). Indeed, our data show that GCF2 expression, not detectable in normal brain, is remarkably induced after the ischemic occlusion. To our knowledge, this is the first report on brain GCF2 expression after ischemia. These findings strongly suggest that the newly created consensus binding site for GCF2 is active, thus leading to an active repression of *EAAT2* expression. These results may be translated into a marked impairment of glutamate uptake in patients affected by this polymorphism and may explain the differences in glutamate concentrations and clinical outcome in patients with mutant and wild-type genotype reported in this study.

This study has several limitations. The size of the patient population was rather small, thus hampering the genotype-phenotype correlation. Second, we cannot rule out that the presence of polymorphisms in other genes, such as genes related to proinflammatory molecules, could affect the progression of the disease and mask the *EAAT2* polymor-



**Figure 5.** GCF2 protein levels in rat brain cortex (A) and striatum (B) from sham-operated rats and 2 and 24 h after permanent middle cerebral artery occlusion. Representative Western blot of five different experiments.

phism effect. However, this study also has some strengths. Patients were studied prospectively with a comprehensive protocol that included advanced multimodal MRI, so we can reasonably rule out the influence of different initial brain injury in our results. Furthermore, glutamate determinations were measured sequentially during the first 3 d, showing that the increase was a result of the cerebral ischemia because they dropped to the normal range at 72 h.

Impaired glutamate transport is known to induce neurotoxicity associated with numerous neurological processes. In the case of *EAAT2*, reductions in its protein expression have been described to take place in ischemia (28), temporal lobe epilepsy (29), Alzheimer's disease (30), Huntington's disease (31), and amyotrophic lateral sclerosis (32, 33) among others. This highlights the importance of the polymorphism described in this paper not only on stroke outcome, the main focus of the present study, but also in other neuropathologies, which deserves further study.

In conclusion, this study has revealed a novel functional polymorphism in the *EAAT2* promoter region and a pattern of regulation that decreases promoter activity. This polymorphism is associated with higher plasma glutamate levels and a clinically relevant trend toward neurological worsening after stroke. These findings may explain the reported failure of glutamate antagonists in human stroke and prompt the use of pharmacogenetics in future clinical trials with drugs blocking or modifying the excitotoxic pathway.

## MATERIALS AND METHODS

### Clinical study

We prospectively studied 101 patients with a nonlacunar hemispheric ischemic stroke admitted within 12 h from the onset of symptoms. 106

healthy control subjects matched by age, sex, and vascular risk factors and who were selected among patients' partners were also studied. Exclusion criteria were unconfirmed acute cerebral infarction by MRI, rapid improvement of symptoms by the time of inclusion, known active seizure or seizure within 6 h before initiation of study, previous stroke with persistent ipsilateral deficits or prior territorial infarction, chronic liver, renal, hematological or immunological diseases, uncontrolled diabetes, and infectious disease in the 15 d before inclusion. The Ethics Committee (Hospital Universitario Josep Trueta) approved the protocol, and the patients or relatives gave informed consent.

At admission, the NIHSS score was evaluated by a certified neurologist and a multiparametric MRI was performed (median time from stroke onset, 4.0 h [2.5, 6.3]). Patients were treated according to published guidelines (34). Early neurological deterioration (END) was defined as an increase  $\geq 4$  points in the NIHSS score between admission and 72 h (13). No patient was lost in follow-up.

Glutamate concentrations were determined in all patients by HPLC in plasma samples obtained on admission (2.1 h [1.3, 3.3] from onset) at 24 and 72 h upon arrival, following the method described elsewhere (4). As previously reported (4), plasma glutamate concentration values of  $>200 \mu\text{mol/L}$  have the highest sensitivity and specificity in predicting progressing stroke.

**Polymorphism analysis.** Genomic DNA was isolated from peripheral blood samples. Polymorphism screening of *EAAT2* promoter was performed by PCR and single-strand conformation polymorphism (SSCP) analysis (35). For patients in which abnormal SSCP patterns were found, PCR products were automatically sequenced. Primers used in  $-181 \text{ A/C}$  polymorphism fragment amplification are described in Fig. 1 B.

### Experimental study

All procedures conformed to the Committee of Animal Care at the Universidad Complutense of Madrid according to European Union and Spanish rules (86/609/CEE and RD223/88).

**Rat astrocyte cultures.** Primary astrocyte cultures were prepared from neonatal (P0) Wistar rat cortex as described previously (36, 37).

**Construction of the plasmid pEAAT2PrLUC.** A 773-bp fragment including  $-742/+31$  of full-length *EAAT2* promoter was amplified by PCR introducing MluI and BglII restriction sites (Fw-MluI: 5'-CTAGACGCGTTTCAGCCGCTCGACTC-3' and Rv-BglII: 5'-ATGAAGATCTCTTGGCGGGGAGCTC-3'). After enzyme digestion and fragment purification, the  $-742/+31$  fragment from wild-type and mutant promoters were fused upstream of the firefly luciferase reporter in pGL3-basic luciferase reporter vector.

**Cell transfection and luciferase reporter gene assay.** Reporter assays were performed on wild-type and mutant *EAAT2* promoter constructions (pEAAT2PrLUC-wt and pEAAT2PrLUC-mut) using the Dual-Luciferase reporter assay system (Promega) with which pRL-thymidine kinase (TK) plasmid (Promega), containing the *Renilla* luciferase gene under control of the TK promoter, was cotransfected as an internal control. In some experiments, this vector was cotransfected with the GCF2 and/or AP-2 plasmids. The pGST/GCF2 plasmid, containing the GCF2 transcription factor, and the SPRSV-AP-2 plasmid, containing the AP-2 transcription factor under the control of RSV promoter and enhancer elements, were gifts from A.C. Johnson (National Institutes of Health, Bethesda, MD) and T. Williams (University of Colorado Health Sciences Center, Denver, CO), respectively. Transient transfection of astrocytes was performed as described previously (38) using Lipofectamine 2000 (Invitrogen). The activities of control *Renilla* luciferase and firefly luciferase were measured in triplicate.

**Permanent pMCAO in rats.** Experiments were performed on male Fischer rats weighing 250–300 g as described previously (39, 40). Rats in which the middle cerebral artery was exposed, but not occluded, served as sham-operated controls.

### Western blot analysis of GCF2 in sham and ischemic rat brain.

Brain homogenates from ipsilateral cortices and striata obtained from rats killed 2 and 24 h after MCAO (40  $\mu\text{g}$  of protein) were loaded and the proteins were size separated in 10% SDS-PAGE (90 mA; reference 36). Proteins were blotted onto a PVDF membrane (GE Healthcare) and incubated with the specific primary monoclonal antibody against GCF2 (BD Transduction Laboratories; 1:250 dilution). Proteins recognized by the antibody were revealed by ECL kit (GE Healthcare).  $\beta$ -actin was used as loading control.

**Statistical analyses.** Results are expressed as percentages for categorical variables and as mean  $\pm$  standard deviation or median (quartiles) for the continuous variables depending on their normal distribution or not. Proportions were compared using the chi-square test or Fisher's exact test as appropriate. Student's *t* test or Mann-Whitney test were used to compare continuous variables between groups. A *p*-value of  $<0.05$  was considered as statistically significant. Statistical analysis was performed using the 11.0.0 version of SPSS software (SPSS Inc).

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