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Plasma Glutamate and Glycine Levels in Patients with Amyotrophic Lateral Sclerosis

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Abstract. *Defective glutamate (Glu) metabolism and glutamate excitotoxicity have been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS). Glycine (Gly), the main inhibitory neurotransmitter, has been shown to potentiate excitatory transmission. In the present study, the levels of Glu and Gly in fasting plasma were measured by high performance liquid chromatography (HPLC) in 20 healthy volunteers and in 65 untreated ALS patients. Increased plasma Glu levels were observed in ALS ($p=0.05$), correlating with longer disease duration ($p=0.03$, beta=0.34) and male gender ($p=0.02$). Furthermore, the increase was found only in the spinal subtype of the disease ($p=0.03$), while in the bulbar subtype, no significant increase was noted. As regards plasma Gly, no difference was observed between patients and controls; however female patients had higher levels than males. The above results are compatible with the "glutamate hypothesis" of ALS and suggest that the spinal and bulbar-onset subtypes of the disease may be biochemically different.*

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by selective degeneration of motoneurons in the spinal cord, brainstem and cortex, clinically manifested by progressive muscular weakness, wasting and spasticity. The precise mechanism that leads motoneurons to death is unknown; however it has been generally accepted that excitotoxicity plays a role, either as a primary action or as an indirect consequence of oxidative stress (1-3). Glutamate (Glu) is thought to serve as the major excitatory neurotransmitter of the corticospinal pathway (4). The chronic loss of glutamate transport (5) that has been observed in ALS and the subsequent deficit in glutamate clearance in the synaptic cleft is considered to result in overstimulation of the N-methyl-D-aspartate (NMDA) receptor and leads to neuronal damage, probably through calcium overload (6).

Although defective Glu metabolism occurs systematically in ALS, the neuropathological changes of the disease are known to be primarily localized in the spinal cord, brainstem and cortex. An imbalance between excitation and inhibition has been proposed to confer selective neuronal vulnerability and mediate cell death (7).

Glycine (Gly) is a classic inhibitory neurotransmitter of the spinal cord and brainstem; it is the neurotransmitter of Renshaw cells, the interneurons that modulate excitation of spinal motoneurons by recurrent inhibition. However, it is also a co-agonist at excitatory NMDA-type glutamate receptors. It has been shown to potentiate glutamatergic neurotransmission by acting on a strychnine insensitive allosteric site of the NMDA receptor, thus increasing the frequency of channel opening. This distinctive action probably accelerates recovery of the receptor from glutamate-induced desensitization (7, 8) and increases the excitotoxic effect of glutamate. Since patches of glycine receptors are localized near postsynaptic glutamate receptors, glycine is positioned to modulate both inhibition and excitation simultaneously (9).

Because of the conflicting results regarding plasma levels of these neurotrasmitters reported by different groups of investigators (10-15), we examined plasma Glu and Gly levels and their possible relation to disease severity and duration.

Patients and Methods

Patients. A total of 85 subjects were included in the study. Informed consent was obtained from each subject and the study protocol had the approval of the scientific committee of our hospital. The study was in accordance with the Declaration of Helsinki ethical guidelines.

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The patient group constituted 65 adult patients with sporadic ALS fulfilling the World Federation of Neurology El Escorial criteria (16, 17) and their functional status was assessed with the Appel score (18). All patients were free of medication. Blood tests for thyroid and parathyroid function, vitamin B_{12} and folic acid, trace elements and heavy metals, immunoelectrophoresis, carcinoembryonic antigen (CEA), alphafetoprotein and β-hexosaminidase A levels, syphilis serology and computed tomography (CT) of the thorax and abdomen were performed for differential diagnosis and exclusion of cases mimicking ALS. No evidence of multifocal conduction block was present on nerve conduction studies. Magnetic resonance imaging (MRI) was also used according to clinical indications for exclusion of cervical myelopathy or intracranial pathology. In most patients lumbar puncture was also performed and a normal biochemical, immmunoelectrophoretic and trace element spinal fluid profile was required for inclusion in the study. Genetic testing was used to investigate abnormal expansion of the CAG repeat in the exon 1 of the androgen receptor gene on the X chromosome, based on clinical suspicion, in order to exclude Kennedy's disease.

The control group constituted 20 healthy volunteers who were subjected to physical examination and were found to be neurologically normal. The clinical characteristics of patients and controls are summarized in Table I.

Methods. Blood samples were obtained after overnight fasting at 08.00-10.00 hours. Sample handling and biochemical assay by high performance liquid chromatography (HPLC) was performed as previously described (19, 20). Briefly, all samples were immediately (within 5 minutes) transferred on ice to the laboratory and deproteinized by centrifugal ultrafiltration through Ultrafree-MC UFC3GC filters (Millipore Corporation, Bedford, Massachusetts, USA). In order to avoid contamination, tubes and pipettes were previously soaked in perchloric acid (6N) for 24 hrs and rinsed 5-6 times with Milli-Q water. All aliquots were stored at –80˚C until determination of Glu and Gly, which was performed by the "Pico-Tag" HPLC method, (Waters, Milford, MA, USA) with pre-column derivatization (phenylisothiocyanate) and ultraviolet/visible (UV) detection at 256 nm (486 UV detector, Waters). The Maxima-820 software (Waters) was used for pump control and peak quantitation. Lyophilized serum from the ERNDIM Quality Control program for amino acid analysis was regularly used to check the method (21).

Statistical analysis. All variables were checked for normality and homogeneity of variances by the Shapiro-Wilk's and Levene's test respectively. Some variables differed significantly from the normal distribution and are presented in terms of median values and quartiles. Fortunately, logarithmic transformation restored normality in these variables and permitted the use of parametric tests. Thus, 2-way analysis of covariance (ANCOVA) (after logarithmic transformation when appropriate) with the diagnostic group and sex as co-factors and age as covariate was used to compare Glu and Gly levels and the Glu/Gly ratio between ALS and controls. In the ALS group, ANCOVA with Newmann-Keuls *post hoc* test was also used, with gender, level of diagnostic confidence (according to El Escorial criteria) and type of ALS (spinal or bulbar) as co-factors and disease duration, age or age of onset as covariates. Multiple regression and Spearman correlation coefficient were also used as appropriate.

Table I. *Clinical and biochemical data of studied groups.*

	Controls	ALS	*P*-value
n	20	65	
Gender (males/females)	12/8	37/28	NS
Age (years)†	60±10.2	58.2±12.1	NS
Age of onset (years)†		56.1±13	
ALS duration (months)‡		17.5 (10–33)	
Type (spinal/bulbar)		45/20	
Diagnostic confidence (possible/probable/ definite)	17/19/29		
Apel score‡		67 (48–90)	
Plasma Glu (μM)‡	32.8 (22.3–41.6)	38.4 (29.5–47.6)	0.05
Plasma Glu/Gly	0.15 (0.1–0.21)	0.19 (0.13–0.28)	0.027
Plasma Gly (μM) †	211.9±63.5	205.3±56.4	NS

†Mean±SD; ‡median (25th-75th percentile). ALS: amyotrophic lateral sclerosis; NS: not significant.

Results

The clinical and biochemical data of patients are summarized in Table I. ANCOVA revealed that patients with ALS, presented with higher plasma Glu levels (Figure 1A). Age, Appel score and the degree of diagnostic confidence during initial evaluation (possible, probable, definite) did not affect plasma Glu level, however the type of ALS did, since the spinal (but not the bulbar) type presented with higher Glu as compared to controls (Figure 1B). Sex also affected Glu only in ALS (Figure 1C), and ALS duration positively affected Glu levels (Figure 1D). As regards plasma Gly, the patients did not differ from the controls, there was no effect of age, ALS duration, type of disease or level of diagnostic confidence. However, in ALS (but not in the controls) females had higher Gly levels than males (221.2±57.1 *vs.* 188.6±46.5 respectively, $p=0.02$). Glu and Gly levels did not correlate between each other significantly in either ALS or the controls. The Glu/Gly ratio was significantly higher in ALS as compared to the controls (Table I) with the spinal type showing higher values than the bulbar subtype and the controls ($p=0.016$). Males in general presented with higher Glu/Gly ratio than females ($p=0.004$)

Discussion

Defective Glu and probably other amino acid metabolism has been repeatedly reported in patients with ALS (10, 12) and attributed possibly to a chronic loss of glutamate transport (5). However these findings have also been criticized mostly based on methodological grounds (22).

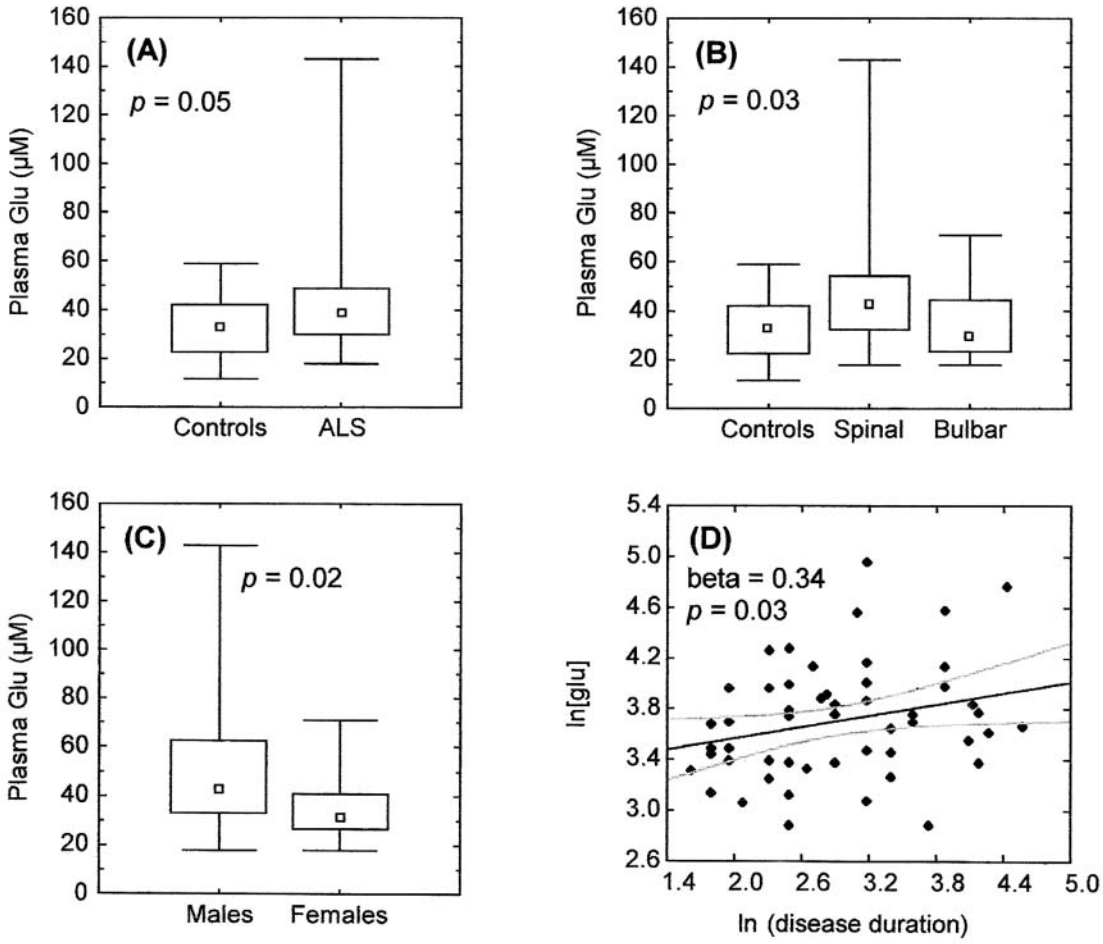

Figure 1. *Plasma levels of Glu in the ALS group were higher as compared to controls (A). The spinal type of the disease (B), male gender (C) and prolonged duration (D) were associated with significantly higher plasma Glu levels. In (A), (B) and (C) box and whiskers plots indicate median values, 25th-75th percentile and range, while the p-value is the result of ANCOVA after logarithmic transformation of original data. In (D) the regression line is shown with 95% confidence interval.*

The best method regarding collection and storage of Glu and other free amino acids is still uncertain. Two major methodological problems have been recognized; the rapid degradation of Glu attributed to its metabolic instability in the biological samples and artifactual *in vitro* increase of Glu due to the addition of acids (such as sulfosalicylic and perchloric) for deproteinization purposes. These acids probably result in hydrolysis of glutamine and subsequent Glu increase (22). In order to avoid the above pitfalls immediate transfer (on ice) of the samples to the laboratory was ensured, facilitated by its very close proximity to our clinic and the use of an ultra filtration method for deproteinization which avoided the addition of any substance to the samples.

The results of the present study were compatible with the glutamatergic hypothesis of ALS. However the glutamate increase was only shown by a subgroup of ALS patients, those with spinal onset. The subgroup of ALS patients with bulbar onset presented with normal plasma Glu levels. Niebroj-Dobosz and Janik (15) reported increased serum glutamate in patients with severely progressing ALS. However it is not clear whether these cases were of spinal or bulbar onset. In a subsequent study, where all the patients except one were of spinal origin, Glu levels were reported to be elevated (23). Previous studies did not describe results in terms of disease subtypes (10-13). Our finding of increased levels of plasma Glu in patients with spinal onset is in agreement with the observation of Camu *et al.* (14). However, we did not confirm their finding of reduced plasma Glu in patients with bulbar onset, although our study included a greater number of patients as well as neurologically normal subjects, instead of patients suffering from other neuropsychiatric disorders, as a control group. Spreux-Varoquaux *et al.* have reported higher

Glu concentrations in the cerebrospinal fluid (CSF) of ALS patients with spinal onset in a large cohort, suggesting either different pathogenetic mechanisms in subtypes of the disease or a reflection of the intensity of cell insult in the spinal cord (24). Excessive synaptic amounts of Glu may be transported to the CSF as well as to the blood and could possibly contribute to the elevated plasma levels of Glu.

The higher Glu concentrations found in patients with longer disease duration could be attributed to the ongoing loss of glutamate transport and uptake.

Male patients had higher Glu concentrations compared to females. This might possibly be related to the slightly higher incidence of the disease in males.

Glycine in the plasma of ALS patients has been reported to be elevated (13, 15), normal (10, 14) or decreased (15). Changes in the plasma glycine levels have been claimed to be inversely correlated with the severity of the disease, being either normal or decreased in severely progressing ALS, while increasing in patients with a lower rate of disease progression (15). In the present study, plasma Gly levels did not differ between patients and controls, nor was there any correlation with age, duration or type of disease. However even normal levels of free glycine in the spinal cord and brainstem could render these central nervous system (CNS) regions susceptible to glutamate neurotoxicity, by preventing desensitization of the NMDA receptors (7). Glutamate receptor density differs between affected and spared motoneurons in ALS, the latter expressing a lower density of NMDA receptors and a higher density of non-NMDA receptors (25-27). Moreover, there is evidence that some NMDA receptors are activated by glycine, but not glutamate (28). Female patients had higher Gly levels than male patients. Since no difference between the two sexes in Gly levels was observed in the control group, this finding might correlate with the higher incidence of bulbar onset in female patients (29).

In conclusion, although no difference was observed in plasma Gly levels between patients and controls, substantial differences between ALS subtypes were found, plasma Glu being increased in the spinal type, while remaining normal in bulbar ALS. This different amino acid profile of patients with spinal onset favors the hypothesis that the two subtypes of of ALS may be biochemically different.

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