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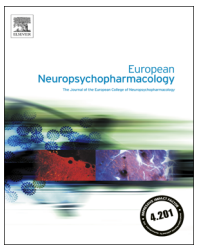

Markers of glutamate signaling in cerebrospinal fluid and serum from patients with bipolar disorder and healthy controls

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Abstract
Glutamate is the major excitatory neurotransmitter in the brain. Aberrations in glutamate signaling have been linked to the pathophysiology of mood disorders. Increased plasma levels of glutamate as well as higher glutamine+glutamate levels in the brain have been demonstrated in patients with bipolar disorder as compared to healthy controls. In this study, we explored the glutamate hypothesis of bipolar disorder by examining peripheral and central levels of amino acids related to glutamate signaling. A total of 215 patients with bipolar disorder and 112 healthy controls from the Swedish St. Göran bipolar project were included in this study. Glutamate, glutamine, glycine, L-serine and D-serine levels were determined in serum and in cerebrospinal fluid using high performance liquid chromatography with fluorescence detection. Serum levels of glutamine, glycine and D-serine were significantly higher whereas L-serine levels were lower in patients with bipolar disorder as compared to controls. No differences between the patient and control group in amino acid levels were observed in cerebrospinal fluid. The observed differences in serum amino acid levels may be interpreted as a systemic aberration in amino acid metabolism that affects several amino acids related to glutamate signaling.

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1. Introduction

Glutamate is the major excitatory neurotransmitter in the brain. Aberrations in glutamate signaling have been linked to psychiatric disorders such as schizophrenia (Javitt and Zukin, 1991), alcohol dependence (Lovinger et al., 1989), and obsessive-compulsive disorder (Rothbaum, 2008). Aberrant glutamate signaling has also been implicated in the pathophysiology of mood disorders. Specifically, increased plasma glutamate levels have been observed in patients with mood disorders compared to healthy controls (Altamura et al., 1993; Mauri et al., 1998), and a recent meta-analysis concluded that patients with bipolar disorder have higher glutamine+glutamate levels in the brain compared to healthy controls, as measured by magnetic resonance spectroscopy (Gigante et al., 2012). Further, a postmortem study showed higher glutamate levels in the frontal cortex of patients with bipolar disorder (Hashimoto et al., 2007). A role of the glutamatergic system in the pathophysiology of bipolar disorder is also supported by findings of altered glutamate receptor expression and glutamate receptor polymorphisms as well as significant effects of mood stabilizers on glutamatergic transmission (Sanacora et al., 2008).

Several of the currently used mood stabilizers have affinity for glutamate receptors (Machado-Vieira et al., 2009). The N-methyl-D-aspartic acid receptor (NMDAR) subtype of glutamate receptors is of particular interest since the non-competitive NMDAR antagonist ketamine has shown antidepressant effects in humans (Berman et al., 2000) as well as in animal models of depression (Duman and Aghajanian, 2012). Moreover, administration of synthetic NMDAR antagonists can also cause psychotic symptoms (Krystal et al., 1994), which is a common feature during manic episodes in bipolar disorder. In line with this, we have shown that kynurenic acid, which is an endogenous antagonist of NMDAR, is increased in cerebrospinal fluid (CSF) of patients with bipolar disorder who have had psychotic episodes (Olsson et al., 2012).

Measurements of peripheral levels of amino acids related to glutamate signaling may improve the understanding of glutamatergic function in psychiatric disorders and how the glutamatergic system is modified by psychoactive medications. However, the biochemical composition in blood does not necessarily translate to the brain biochemistry influencing neural operation. Analyses of peripheral biomarkers should therefore preferably be paralleled by analyses of neurochemicals in CSF.

The primary aim of this study was to test if serum concentrations of amino acids correlate with CSF concentrations and thus provide information on brain glutamatergic signaling. A secondary aim was to test if amino acid sampling might serve as a marker of pharmacological treatment effects related to changes in glutamatergic signaling. To these ends, we determined serum levels of glutamate, glutamine, glycine, L-serine, and D-serine from mood stabilized bipolar disorder patients and sex- and age-matched healthy controls. In a subset of these patients and controls, we also determined these amino acids in CSF.

2. Experimental procedures

The study population was recruited from the St. Göran bipolar project, which provides assessment, treatment, and follow-up of patients with bipolar disorder within the Northern Stockholm Mental Health Service and serves as a basis for research into bipolar disorder. The methodology has previously been outlined in detail (Ekman et al., 2010; Jakobsson et al., 2012; Ryden et al., 2009). A total of 215 patients with bipolar disorder and 112 healthy controls were included in this study. All patients met the DSM-IV-TR criteria for bipolar disorder. Healthy controls matched by sex and birth date were selected randomly from the national population register by Statistics Sweden (www.scb.se). These control subjects were living in the same catchment area as the patients. Exclusion criteria for controls were (1) any on-going psychiatric or neurological disorder; (2) current treatment with any psychotropic drugs; (3) past bipolar disorder, schizophrenia, recurrent depression, or other psychiatric disorder leading to functional disability; (4) a first-degree relative with schizophrenia or bipolar disorder; (5) subjects presenting conditions that precluded magnetic resonance imaging of the brain (e.g., metal implants, shrapnel and certain heart operations).

Prior to the study, all subjects were provided with verbal and written information about the study and about potential risks and benefits of study participation. All subjects consented orally and in writing to participate in the study. The Regional Research Ethics Board in Stockholm, Sweden approved the study.

2.1. Assessment of clinical variables

Patients were assessed by a psychiatrist or resident in psychiatry using the Affective Disorders Evaluation (ADE), which is a standardized protocol adapted from the Systematic Treatment Enhancement Program of Bipolar Disorder (STEP-BD) (Sachs et al., 2003). The ADE guides the interviewer through a systematic assessment of the patient's current and past mental state and provides a diagnosis according to DSM-IV criteria. The number of lifetime affective episodes and their characteristics are documented. Other modules assess alcohol and drug misuse, violent behavior, childhood history, family history, treatment history, reproductive history and somatic illnesses. Suicide attempt was defined as a deliberate and serious self-injury, including intoxication with medication. The ADE was complemented with the M.I.N.I Neuropsychiatric interview (Sheehan et al., 1998). The final diagnosis was established using LEAD (Longitudinal observation by Experts using All Data) (Spitzer, 1983) and confirmed by a consensus panel of 2–4 experienced clinicians. Inclusion criteria for this sub-study were bipolar I or II diagnosis. Psychosocial functioning was assessed using the clinician rated Global Assessment of Function (GAF) scale (Guy, 1976; Luborsky, 1962). Mood stability was determined by the treating physician's overall diagnostic judgment, but was complemented with the Montgomery-Åsberg Depression Rating Scale (MADRS) and the Young Mania Rating Scale (YMRS) to assess depressive and manic symptom severity, respectively.

2.2. Measurement of serum and CSF levels of mature glutamine, glycine, glutamate, L-serine and D-serine

Serum samples were collected from fasting subjects between 8:00 and 9:00 am. The samples were centrifuged on site and stored at −80 °C until delivered by courier mail, frozen on dry ice, to Chiba University, Japan, for analysis. CSF sampling was performed by lumbar puncture between 9:00 and 10:00 am on the same day as the serum sampling. The spinal needle was inserted into the L3/L4 or L4/L5 interspace, and a total volume of 12 mL of the CSF was collected, gently inverted to avoid gradient effects, and divided into 1.0–1.6 mL aliquots that were stored at −80 °C pending analysis.

Measurement of total, D- and L-serine levels in serum and CSF were carried out using a column-switching high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) as previously reported (Fukushima et al., 2004; Hashimoto

et al., 2005b). Measurement of glycine, glutamine and glutamate was carried out using a HPLC system with fluorescence detection, as previously reported (Hashimoto et al., 2005a).

3. Statistical analyses

SPSS version 20.0 (IBM Corp.) was used to analyze data. Amino acid concentration variables were inspected for skewness and analyzed using the Kolmogorov-Smirnov test, and 10log transformations were used when needed to normalize distributions. Ratios were arctan-transformed prior to analysis. A linear regression model was computed for each amino acid concentration, where the amino acid served as dependent variable, and patient/control status, sex, age and BMI served as independent variables. Spearman correlations were used to study bivariate associations between amino acid concentration and other continuous variables. Multivariate linear regression modeling was used to evaluate the effect of psychotropic medication on amino acid levels. The assumptions of the models (linearity, homoscedasticity and lack of multicollinearity) were checked. False discovery rate was used to control for multiple comparisons as indicated in the results (Benjamini and Hochberg, 1995) and $p<0.05$ was considered statistically significant.

4. Results

4.1. Patient-control comparisons

Serum samples from 215 patients with bipolar disorder and 112 healthy controls (Table 1) were analyzed. In this group, CSF samples were available from 132 patients and 87 controls that were analyzed. Demographics and clinical characteristics of the patient population are presented in Table 1. We determined the serum and CSF concentrations of five amino acids: glutamine, glycine, glutamate, D-serine and L-serine. In addition, the D-serine/L-serine, L-serine/glycine and glutamine/glutamate ratios were calculated. The Kolmogorov-Smirnov test was used to determine that data was normally distributed. The test indicated non-normal distribution for serum glycine, glutamate, D-serine and L-serine, as well as CSF glycine, glutamate and D-serine. Hence, these variables were log-transformed prior to further analyses.

As shown in Table 2, serum levels (adjusted for age, sex and BMI) of glutamine, glycine and D-serine were significantly higher in patients with bipolar disorder as compared to controls. Serum L-serine levels were lower in the patient group. These differences remained significant when adjusting for multiple testing. Hence, the patient group had higher D/L-serine ratio ($t=5.0$, $p<5.2\times10^{-7}$) but lower L-serine/glycine ratio ($t=-6.61$, $p<2.3\times10^{-10}$) than the control group. The glutamine/glutamate ratio did not differ between the groups.

In CSF, the mean concentration of glutamine (adjusted for age, sex, BMI and height) was significantly higher in bipolar disorder patients than in healthy controls ($p=0.026$, Table 3). However, this difference did not survive correcting for multiple testing. We found no other significant differences between patients and controls with respect to the mean CSF amino acid concentrations or ratios.

Smoking status has previously been associated with both serum and CSF amino acid levels (Luykx et al., 2013) and smoking was more common in the patient group. However, adding smoking status as a co-variate to the regression models did not explain the observed differences between the patient and control group.

The patients included in the study were mood stabilized but not consistently euthymic as defined by a MADRS score below 14. Some patients suffered from lingering depressive symptoms with MADRS > 13. Previous work has shown associations between acute depression and altered plasma levels of glutamate, glutamine, glycine and L-serine (Mitani et al., 2006). We therefore tested the association between MADRS scores and amino acid levels using Spearman correlations. Only serum L-serine was significantly associated with MADRS score ($r=-0.15$, $p=0.039$) and this result did not survive correcting for multiple testing.

4.2. Amino acid associations with medications

In order to identify possible glutamatergic markers for mood stabilizing medication, we conducted a post-hoc analysis where we analyzed the association of current treatment with lithium, valproate, or lamotrigine with serum or CSF amino acid levels using multiple linear regression models. We also included sex (female as reference), age, height (CSF only), BMI, GAF score, total number of episodes, and current treatment with antidepressants and antipsychotics as co-variates in the model to control for potential confounders.

As shown in Table 4, regression models that included statistically significant effects for at least one of the mood stabilizers were found for serum glycine and L-serine, as well as CSF glutamine, D-serine and L-serine. Briefly, serum glycine and L-serine, as well as CSF glutamine and D-serine levels were positively associated with lithium and valproate treatment. CSF L-serine levels were positively associated with all three mood stabilizers included in the model. These associations remained significant when correcting for potential confounders. Correction for multiple comparisons was not performed for these exploratory post-hoc analyses.

All patients included in the study were in a stable mood at sampling but some patients experienced residual symptoms. Thus, YMRS and MADRS scores were added to all models but this did not explain the observed associations of mood stabilizer treatment and amino acid levels.

5. Discussion

This is the first study to investigate the glutamatergic system in bipolar disorder by assessing both serum and CSF concentrations of glutamine, glutamate, glycine, L-serine and D-serine in mood-stabilized bipolar patients and healthy controls. We found that the serum levels of glutamine, glycine and D-serine were significantly higher in clinically stable patients with bipolar disorder as compared to healthy controls, while L-serine serum levels were significantly lower in the patient group. This pattern was not mirrored in CSF where no statistically significant differences were found between the patient and control group.

Table 1 Characteristics of the study sample.

	Controls		Bipolar disorder	
Sex (male/female)	50/62		Sex (male/female)	82/133
	Median	IQR	Median	IQR
Age (years)	35	27-45	36	28-48
BMI	23.3	21.6-25.4	24.7	22.1-27.8
	N	%	*N*	%
Smoker[a]	16	14	67	34
Diagnosis			*N*	%
Bipolar disorder type I			108	50
Bipolar disorder type II			82	38
Not otherwise specified			25	12
Previous psychosis			106	49
Alcohol or substance use disorder[b]			69	32
Anxiety disorder[c]			91	42
ADD[d]			28	13
History of suicide attempt			81	38
Clinical data			Median	IQR
Age first symptoms[e]			16	13-24
Depressive episodes[f]			10	5-20
Hypomanic episodes			2	0-6
Manic episodes			1	0-2
Mixed episodes			0	0
GAF			67	60-75
MADRS[g]			4	0-11
YMRS[h]			0	0-2
Medication			*N*	%
Lithium			129	60
Valproate			28	13
Lamotrigine			47	22
Antidepressants			81	38
Antipsychotics			56	26

[a]Missing data for 15 individuals in the patient group.
[b]Missing data for 3 individuals in the patient group.
[c]Missing data for 1 individuals in the patient group.
[d]Missing data for 4 individuals in the patient group.
[e]Missing data for 2 individuals in the patient group.
[f]Missing data for 3 individuals in the patient group.
[g]Missing data for 31 individuals in the patient group.
[h]Missing data for 31 individuals in the patient group.

Previous work using magnetic resonance spectroscopy has shown an association between bipolar disorder and higher glutamine+glutamate levels in the brain (Gigante et al., 2012). Our finding of higher glutamine levels in serum in patients as compared to controls thus partly concur with this observation, though we did not observe increased glutamine or glutamate in CSF. Further, it is not clear if the observed differences in amino acid levels are specific to bipolar disorder. Other studies have shown decreased D-serine levels in both serum and CSF from patients with schizophrenia (Hashimoto et al., 2003, 2005b). However, increased serum levels of D-serine (Ohnuma et al., 2008) and total serine (Sumiyoshi et al., 2004) have also been observed. With respect to bipolar disorder, one previous study using postmortem brain samples found no differences in L-serine or D-serine as compared to a control group (Hashimoto et al., 2007). The present findings of increased peripheral D-serine and decreased L-serine levels suggest an aberration in serine metabolism in patients with bipolar disorder. Previous studies in unipolar depression have shown both decreased (Mitani et al., 2006) and increased (Sumiyoshi et al., 2004) peripheral serine levels.

There are no previous reports on glycine levels in bipolar disorder, but decreased CSF glycine levels were found in a mixed unipolar and bipolar cohort (Frye et al., 2007). Further, associations between serum glycine levels and negative symptoms (Hons et al., 2010) and sensory gating (Heresco-Levy et al., 2007) have been reported in schizophrenia. These studies suggest that serum levels of glycine provide some information on brain function. When it comes

Table 2 Serum amino acid concentrations.

Amino acid	Controls		Bipolar disorder		Analysis				
	Mean	SD	Mean	SD	Beta	*t*	df	*p*	*q*[a]
Glutamine (μM) [b,c]	507	65.5	523	75.9	0.14	2.61	1	0.010	0.025
Glycine [d] (μM) [e,b]	236	62.8	256	78.3	0.12	2.23	1	0.027	0.034
Glutamate [d] (μM) [e,b,c]	54	19.1	58.4	24.9	0.02	0.46	1	0.65	0.65
D-serine[d] (μM) [c]	1.21	0.27	1.30	0.38	0.13	2.26	1	0.024	0.034
L-serine [d] (μM) [b]	130	28.1	115	22.9	−0.29	−5.37	1	1.5×10^{-7}	7.5×10^{-7}

Serum amino acid concentrations in 215 bipolar disorder patients and 112 sex and age matched healthy controls. Differences between patients and controls were analyzed with linear regression using age, sex and BMI as covariates.
[a]*p*-Value adjusted for false discovery rate.
[b]Significant effect of sex.
[c]Significant effect of BMI.
[d]Log transformed prior to statistical analysis.
[e]Significant effect of age.

Table 3 Cerebrospinal fluid amino acid concentrations.

Amino acid	Controls		Bipolar disorder		Analysis				
	Mean	SD	Mean	SD	Beta	*t*	df	*p*	*q*[a]
Glutamine (μM) [b,c,d]	499	65.6	516	82.5	0.13	2.24	1	0.026	0.13
Glycine [e] (μM)[b,f]	7.6	3.5	8.2	4.4	0.06	0.92	1	0.36	0.53
Glutamate [e] (μM) [b]	0.68	0.19	0.75	0.38	0.09	1.44	1	0.15	0.38
D-serine[e] (μM)	1.77	0.38	1.84	0.42	0.06	0.81	1	0.42	0.53
L-serine (μM)	24.6	4.4	24.6	5.2	−0.02	−0.24	1	0.81	0.81

Cerebrospinal fluid amino acid concentrations in 135 bipolar disorder patients and 87 sex and age matched healthy controls. Differences between patients and controls were analyzed with linear regression using age, sex, BMI, and height as covariates.
[a]*p*-Value adjusted for false discovery rate.
[b]Significant effect of age.
[c]Significant effect of sex.
[d]Significant effect of BMI.
[e]Log transformed prior to statistical analysis.
[f]Significant effect of height.

Table 4 Mood stabilizer treatment and amino levels.

Regression model	Lithium			Valproate			Lamotrigine		
Serum	*β*	*p*[a]	*p*[b]	*β*	*p*[a]	*p*[b]	*β*	*p*[a]	*p*[b]
Glycine[c]	0.17	0.019	0.005	0.39	1.2×10^{-8}	3.4×10^{-8}	0.09	0.16	0.10
L-serine[c]	0.20	0.007	0.005	0.19	0.010	0.002	0.06	0.41	0.45
CSF									
Glutamine	0.26	0.004	0.005	0.51	4.2×10^{-8}	2.8×10^{-8}	0.11	0.18	0.098
D-serine[c]	0.32	4.9×10^{-4}	4.3×10^{-4}	0.45	1.0×10^{-6}	2.0×10^{-6}	0.15	0.083	0.067
L-serine	0.27	0.005	0.006	0.35	2.0×10^{-4}	1.0×10^{-4}	0.21	0.017	0.003

Multiple linear regression analysis for each amino acid using lithium, valproate and lamotrigine as explanatory variables.
[a]Unadjusted *p*-values.
[b]*p*-Values adjusted for sex (female as reference), age, BMI, height (for CSF measures), GAF, number of episodes, use of antidepressants and antipsychotics.
[c]Log transformed prior to statistical analysis.

to unipolar depression, there have been reports of both increased (Mitani et al., 2006) and unaltered (Maes et al., 1998) blood glycine levels.

It is not clear whether changes in amino acid levels are a trait marker or dependent on mood state at sampling. A number of patients in our study cohort had lingering depressive symptoms. However, we found no significant associations between amino acid concentrations and MADRS scores, which suggests that altered amino acid levels can be found in euthymic as well as depressed or manic patients.

With the exception of L-serine, the observed differences in amino acid levels between bipolar disorder patients and healthy controls were relatively small. Further, none of these observations were mirrored in CSF. Interestingly, a recent meta-analysis of studies measuring NMDA-receptor co-agonists in schizophrenia found consistently increased serine levels in blood across studies, but could not verify any significant findings in CSF (Brouwer et al., 2013). Taken together, the present study and previous work do not present a clear picture on changes in blood or CSF amino acid concentrations, with relevance for glutamatergic signaling, in bipolar disorder, schizophrenia or major depression.

There are several possible explanations for the discrepancy between serum and CSF measures of amino acid levels. CSF concentrations of glycine and L-serine are normally around 10% of the blood levels (Hawkins et al., 2006). For glutamate, the CSF proportion is even lower whereas glutamine is present in almost the same concentration as in blood. The reason is thought to be an active transport of amino acids from CSF to blood via the blood-brain barrier (Hawkins et al., 2006). This adds complexity when extrapolating from CSF amino acid levels to brain tissue levels.

It should also be noted that glutamine, glutamate, L-serine and glycine perform numerous roles in metabolic and synthesis pathways both centrally and peripherally. Thus, the observed differences in amino acid levels could be related to changes in cellular metabolism rather than glutamatergic signaling. Indeed, mitochondrial dysfunction and impaired cellular metabolism have been implicated in bipolar disorder by findings from, e.g., magnetic resonance spectroscopy studies (Stork and Renshaw, 2005). Changes in D-serine on the other hand may be more directly linked to altered glutamatergic neurotransmission (Pollegioni and Sacchi, 2010) as its main role appears to be as a signaling molecule.

In this study, potential confounding effects of psychotropic medication should be considered. Indeed, serum glycine levels were positively correlated with both lithium and valproate treatment. Thus the observed difference between patients and controls may be explained by current treatment with mood stabilizers. Valproate has previously been associated with increased serum levels of glycine in children (Castro-Gago et al., 1990). However, negative findings on plasma glycine and valproate treatment in adults have also been published (Verity et al., 1983). Treatment with valproate has previously been demonstrated to increase the CSF levels of glutamine (Akiyama et al., 2012; Perry et al., 1976; Scholl-Burgi et al., 2008). This finding was confirmed in the current study, where valproate treatment was positively correlated with CSF levels of glutamine. It has also been described previously that valproate treatment increases the CSF levels of serine, which was also observed in this study (Akiyama et al., 2012). Although, the effect sizes of lithium treatment in relation to amino acid levels were smaller than valproate treatment, lithium treatment was also associated with increased CSF levels of glutamine, D-serine and L-serine. The effect of lithium on glutamate levels has previously been tested in experimental animal models, where lithium treatment increased glutamate release in monkey and mouse cerebral cortex slice preparations (Dixon et al., 1994) and also increased glutamine levels on a trend level (Vargas et al., 1998).

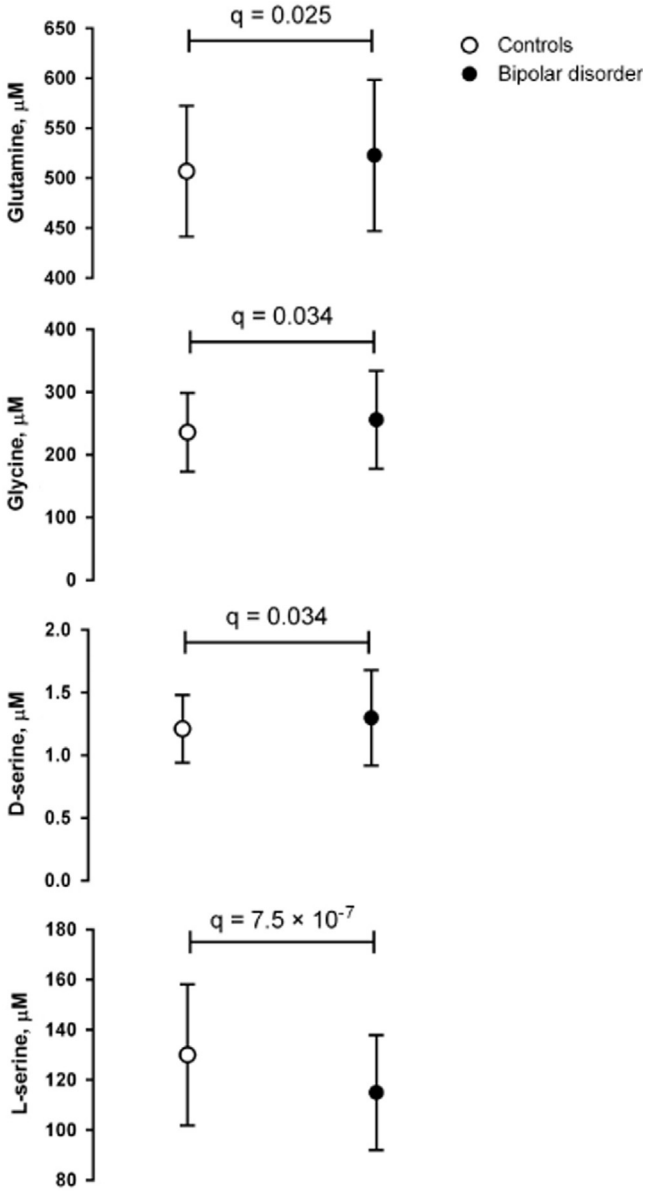

Figure 1 Serum amino acid levels in persons with bipolar disorder and healthy controls. Serum concentrations (mean ± SD) of glutamine, glycine, D-serine and L-serine in healthy controls (n=112) and bipolar disorder patients (n=215). Linear regression was used for comparisons between patient and controls while controlling for age, sex and BMI. Q-values adjusted for multiple comparisons using the false discovery rate method.

The present study includes data from a relatively large group of patients with bipolar disorder in a stable mood. Serum and CSF samples were handled similarly and analyzed in parallel, indicating that differences between the samples are unlikely to be caused by methodological factors. Further, we used a population-based control group that was matched for age and sex in order to minimize bias caused by a non-representative control group. However, the cross-sectional design of the present study does limit the conclusions that can be drawn on medication effects on amino acid levels. The associations between current medication and amino acid levels need to be corroborated in longitudinal studies. In addition, the naturalistic setting of the study means that many patients are treated with more than one drug, which makes it more difficult to draw firm conclusions on the specific effects of different types of medications. The lack of an un-medicated patient group also limits the conclusions that can be drawn with respect to medication effects. In addition, the naturalistic design of the present study means that a significant proportion of the patients were comorbid for other psychiatric disorders such as anxiety disorders or attention deficit disorder.

5.1. Conclusions

Serum measures of glutamine, glycine, L-serine and D-serine were associated with a diagnosis of bipolar disorder. In CSF, no statistically significant differences that survived correction for multiple testing between patients and controls were observed. Taken together, the results support the notion of a systemic aberration in amino acid metabolism that affects several amino acids related to glutamate signaling. Further, treatment with mood stabilizers was associated with amino acid levels, particularly in CSF. This supports previous work where interaction between, e.g., lithium and brain glutamate signaling has been demonstrated. Further work is needed to determine the mechanism of the observed differences in amino acid levels, both in relationship to bipolar disorder and the effects of psychotropic medication Figure 1.

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Contributors

E. Pålsson, K. Södersten, J. Jakobsson, C. Sellgren, H. Ågren and M. Landén designed the study. C.-J. Ekman, C. Sellgren and M. Landén acquired the data. K. Hashimoto and Y. Fujita performed the amino acid measurements. E. Pålsson and J. Jakobsson undertook the statistical analysis of the data. Authors Pålsson E. and Södersten K. managed the literature searches and author E. Pålsson wrote the first draft of the manuscript. All authors contributed to the final draft of the article and approved its publication.

Conflict of interest

The authors declare no conflict of interest.

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