Alterations in serum amino acid profiles in children with attention deficit/hyperactivity disorder

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Received December 29, 2020; Accepted February 15, 2021

DOI: 10.3892/br.2021.1423

Abstract. The objective of the present study was to evaluate the circulating serum amino acid levels in children with attention deficit/hyperactivity disorder (ADHD). A total of 71 children with untreated ADHD and 31 neurotypical controls aged 7-14 years old were examined. Serum amino acid levels were evaluated using high-performance liquid chromatography (HPLC) with UV-detection. Laboratory quality control was performed with reference materials of human plasma amino acid levels. The obtained data demonstrated that children with ADHD were characterized by 29, 10 and 20% lower serum histidine (His), glutamine (Gln) and proline (Pro) levels compared with neurotypical children, respectively. In contrast, circulating aspartate (Asp), glutamate (Glu) and hydroxyproline (Hypro) levels exceeded the respective control values by 7, 7 and 42%. Correspondingly, the Gln-to-Glu and Pro-to-Hypro ratios were 28% and 49%, respectively, lower in ADHD cases compared with the controls. Total Gln/Glu levels were also significantly lower in ADHD patients. No significant group differences were observed between the groups in the other amino acids analyzed, including phenylalanine. Multiple linear regression analysis revealed significant associations between circulating serum Gln, lysine (Lys) (both negative) and Glu (positive) levels with total ADHD Rating Scale-IV scores. The observed alterations in Pro/Hypro and Gln/Glu levels and ratios are likely associated with the coexisting connective tissue pathology and alterations in glutamatergic neurotransmission in ADHD, respectively. Altered circulating levels of His, Lys and Asp may also be implicated in ADHD pathogenesis. However, further *in vivo* and *in vitro* studies are required in order to investigate the detailed mechanisms linking amino acid metabolism with ADHD pathogenesis.

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Key words: attention deficit/hyperactivity disorder, hydroxyproline, glutamine, lysine, neurodevelopmental disorder

Introduction

Attention deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by inattention and/or hyperactive-impulsivity that interferes with brain functioning or development. While contradictory, the existing data demonstrate that the prevalence of ADHD may be as high as 5.3% and 2.5% in children/adolescents and adults, respectively (1). The effects of ADHD have a significant impact on the social life of patients throughout the entirety of their lives, starting from disruptive behavior, resulting in poor performance in standardized tests and impacted social interactions, which may lead to criminal behavior, substance abuse, lack of motivation, school exclusion and subsequent effects on professional development in adulthood (2). In addition, ADHD has also been found to be associated with lower a health-related quality of life (3). Taken together, these impairments result in high ADHD-associated economic costs (4).

ADHD is characterized by complex alterations in the neurobiology and neurochemistry, with impaired dopamine and norepinephrine signaling being the most prominent (5). Previously, it was also demonstrated that altered glutamatergic neurotransmission is involved in ADHD pathogenesis (6). Therefore, unraveling the potential underlying mechanisms implicated in ADHD pathogenesis is essential for improving our understanding of the disorder and further development of management strategies (5).

Amino acids serve a significant role in brain development and functioning (7). In particular, certain amino acids or their precursors, including glutamine, glutamate and γ aminobutyric

acid, are well-established to be involved in neuronal signaling as neurotransmitters (8). Correspondingly, disruption of amino acid metabolism results in significant neurological disorders, particularly in early ontogenesis (9).

Given the role of an altered neurochemistry in ADHD pathogenesis as well as the role of amino acids in neurodevelopment, it is posited that dysregulated amino acid metabolism may significantly interfere with ADHD. However, the existing data is rather contradictory. An earlier study by Bornstein *et al* (10) found significantly lower plasma levels of phenylalanine (Phe), tyrosine, tryptophan (Trp), His and isoleucine in patients with ADD compared with the healthy controls. Zavala *et al* (11) showed there was a significant decrease in plasma Phe and glutamine (Gln) levels, whereas plasma glycine (Gly) levels were found to be elevated in patients with ADHD. Improvement in ADHD symptoms was positively associated with tyrosine, Phe and Trp levels (12). At the same time, no significant alterations in blood and urinary levels of Trp, tyrosine and Phe levels were observed in children with ADHD (13). In view of these inconsistencies, as well as the use of amino acid supplementation in ADHD management, precise analyses of amino acid profiles in ADHD is required and may assist in reconciling these contrasting results. Thus, the objective of the present study was to evaluate the levels of circulating serum amino acid levels in children with ADHD.

Materials and methods

The present study was performed in accordance with the ethical principles of the Declaration of Helsinki and its amendments (14). The protocol of the present study was considered and approved by the Local Ethics Committee of Yaroslavl State University (Yaroslavl, Russia). Parents or legal representatives signed an informed consent forms for participation of their children in the present study prior to investigation. Examination and blood sample collection was performed only in the presence of the parents/guardians.

A total of 71 children (54 boys, 17 girls) diagnosed with ADHD aged 7-14 years old (8.4±2.6 years old) were enrolled in the present study. The diagnosis of ADHD (ICD-10: F90.0) and other neuropsychiatric disorders (exclusion criteria) were based on the clinical records of the outpatient department. ADHD was defined according to ICD-10 criteria, including inattention, hyperactivity and impulsivity (≥3 symptoms of each) (15). Only patients that did not take any specific treatments for ADHD were included in the study, in order to avoid the confounding effects of any potential side effects of pharmacological treatment on amino acid metabolism.

In addition, 31 age (8.0±2.9 years old; age range 7-14 years old) and sex-matched neurotypical children (24 boys, 7 girls) were also examined, and used as the control group. The absence of neuropsychiatric disorders was confirmed during annual medical examinations. No significant group differences in age (P=0.183) or sex (P=0.885) were observed between the groups.

Children and their parents were invited to participate in the study during the annual medical examinations. A total of 35% of all contacted subjects refused to take part in the study (36 out of 102 children and parents).

The parents of all the examined children completed the ADHD Rating Scale-IV for additional verification of the ADHD diagnosis (16). Total ADHD Rating Scale-IV scores in the ADHD patients exceeded those in neurotypical children by a factor of >2 (14.9±9.4 vs. 7.0±5.4, P<0.001).

Whole blood samples were collected in the morning and after overnight fasting by cubital vein venipuncture using 9-ml Vacuette® tubes (Greiner Bio-One International AG). The samples were subsequently centrifuged for 10 min at 1,600 x g at room temperature to obtain blood serum that was stored in Eppendorf tubes at -18˚C until required for analysis.

Evaluation of serum levels of alanine, arginine (Arg), asparagine, Asp, citrulline, glutamine (Gln), glutamate (Glu), Gly, histidine (His), hydroxyproline (Hypro), isoleucine, leucine, lysine (Lys), methionine, ornithine, Phe, proline (Pro), serine, taurine, threonine, Trp, and Val was performed by high-performance liquid chromatography (HPLC) with UV-detection at PerkinElmer S200 (PerkinElmer, Inc.) using a reverse phase Pico Tag Column for Free Amino Acid Analysis (3.9x300 mm) C18 (EMD Millipore).

Precolumn derivatization with phenylisothiocyanate reagent containing 7:1:1:1 (v/v) methanol:triethylamine:water:phenylisothicyanate was performed prior to the analysis. Analysis was performed with aqueous sodium acetate and acetonitrile gradient mode with UV-detection at 240 nm. The commercially available ClinCal® Plasma Calibrator, lyophil., for Amino Acids (lot. no. 10213; ClinCheck) calibrators were used for HPLC-system calibration.

Laboratory quality control was routinely performed with reference materials of human plasma amino acid levels using ClinChek® Plasma Control, lyophil., for Amino Acids, Levels I (cat. no. 10280) and II (cat. no. 10281). The obtained values for all amino acids fitted the certified control range specified by the manufacturer (ClinCheck). The recovery rates for the studied amino acids varied from 94-109%.

Serum amino acid concentrations are expressed as μmol/l. In addition, total glutamatergic metabolite concentration (Glx), defined as a sum of Glu and Gln levels, was calculated as described previously (17). The obtained values of serum Gln, Glu, Hypro and Pro were used for calculating the Gln/Glu and Pro/Hypro ratios.

Statistical analysis was performed using Statistica version 10.1 (Statsoft, Inc.). Evaluation of data distribution performed using a Shapiro-Wilk test revealed skewed distribution of data on amino acid levels in the study groups. Therefore, the median and the respective interquartile range (IQR) boundaries were used as descriptive statistics. Raw data were log-transformed for subsequent processing. Group comparisons were performed using analysis of covariance (ANCOVA) with adjustment for age and sex as covariates and subsequent Bonferroni adjustments. Multiple linear regression was performed in order to evaluate the relative association between serum amino acid levels (independent predictors) and ADHD (dependent variable) after adjustment for age and sex. Correlation analysis was performed using a Spearman's rank correlation coefficient. P<0.05 was considered to indicate a statistically significant difference, whereas 0.05<P<0.1 was considered nearly significant.

Results

The obtained data demonstrate that ADHD is associated with altered amino acid profiles in children. Evaluation of serum

Table I. Essential amino acid levels in the serum of ADHD cases and neurotypical controls.

Amino acid	Control, μM[a]	ADHD, μM[b]	F-value	P-value
Histidine	85.0 (50-120.6)	60.7 (45.2-94.6)	3.140	0.081
Isoleucine	56.1 (47.6-63.9)	54.1 (43.5-70.8)	0.029	0.923
Leucine	118.6 (103.6-133.7)	117.5 (96.2-138.5)	0.105	0.862
Lysine	170.6 (147.5-198.4)	161.5 (120.8-212.1)	1.242	0.360
Methionine	54.2 (46.2-69.1)	51.6 (44.1-60.2)	0.416	0.396
Phenylalanine	55.8 (47.5-68.2)	57.8 (48.4-66.4)	0.009	0.923
Threonine	108.9 (90.5-129)	111.4 (86.8-143.1)	0.768	0.605
Tryptophan	56.5 (43.3-70.6)	58.1 (44.6-71.5)	0.011	0.794
Valine	167.2 (136.9-212.3)	167.2 (128-223.4)	0.060	0.907

[a]Data are presented as the median (inter-quartile range).[b]ADHD, attention deficit/hyperactivity disorder.

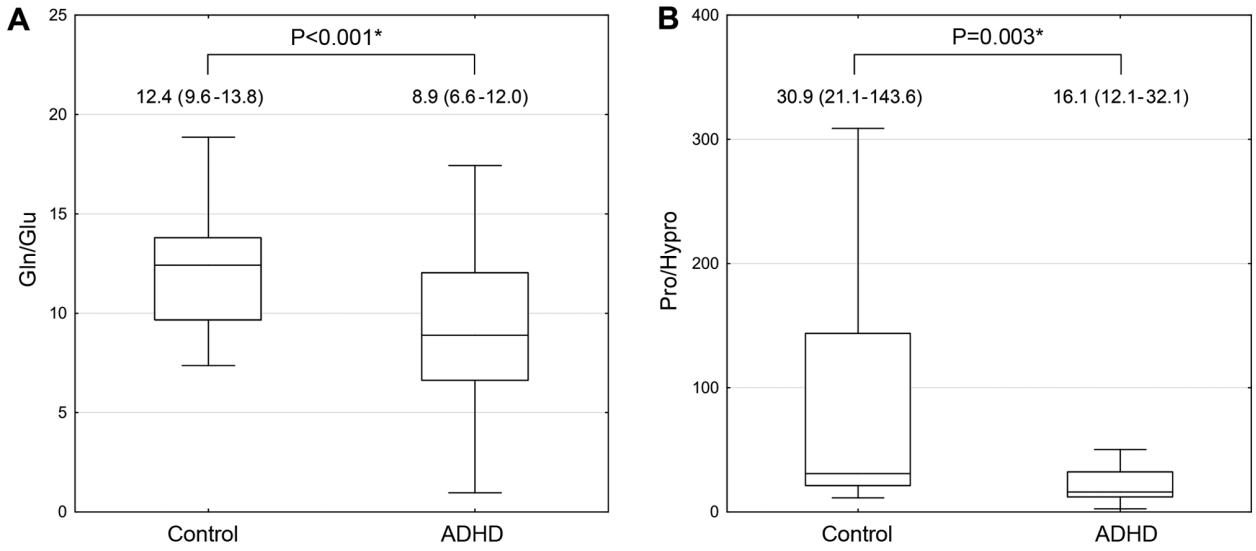

Figure 1. Serum Gln/Glu and Pro/Hypro ratios in children with ADHD compared with the healthy controls. (A) Serum Gln/Glu ratio. (B) Serum Pro/Hypro ratio. Data are expressed as the median (line), inter-quartile ranges (box) and non-outlier ranges (whiskers). *P<0.05. Gln, glutamine; Glu, glutamine; Pro, proline; Hypro; hydroxyproline.

essential amino acids revealed 29% lower His levels in ADHD patients compared with the neurotypical controls (Table I). No significant group differences in other essential amino acids levels were observed.

Greater differences were observed in the levels of conditionally essential and non-essential amino acids (Table II). Particularly, serum Asp and Glu levels were 7% higher compared with the healthy controls, although the difference was significant only in the case of Glu levels. In contrast, serum Gln and Pro concentrations were 10% and 20% lower in ADHD cases compared with the neurotypical controls, respectively. In corroboration with the overall decrease in serum Gln levels, total Glx concentration (Glu+Gln) was also 12% lower in children with ADHD [425.5 (351.5-533.6) μM] compared with the control values [483.1 (406.0-577.4) μM] (F=4.007; P=0.048).

Amongst the amino acid derivatives analyzed, only serum Hypro concentrations were significantly elevated, being 42% higher in ADHD cases compared with the healthy controls.

Given the observed group differences between serum Gln and Glu levels, as well as the levels of Pro and its derivative Hypro, Gln-to-Glu and Pro-to-Hypro ratios were evaluated (Fig. 1). The obtained data demonstrate that Gln/Glu values in ADHD cases were 28% lower compared with the healthy children (F=14.202). In turn, ADHD patients had almost 2-fold lower levels of Pro/Hypro levels compared with the neurotypical controls (F=8.936).

Correlation analysis demonstrated that serum Hypro and Glu concentrations were correlated significantly (r=0.270; P=0.006) and nearly significantly (r=0.194; P=0.051) with total ADHD-RS scores, respectively. Concomitantly, circulating Gln levels (r=-0.207; P=0.037) as well as Gln/Glu ratio (r=-0.376; P<0.001) were characterized by a significant direct correlation with the latter.

Multiple regression analysis was also performed in order to determine if there was an independent association between the serum amino acid levels and total ADHD-RS scores (Table III). In a crude model incorporating all amino acids analyzed, serum

Table II. Conditionally essential and non-essential amino acid levels the serum of children with ADHD and healthy controls.

Amino acid	Control, μM[b]	ADHD, μM[b]	F-value	P-value
Alanine	323.5 (248.3-395.5)	342.7 (298.1-401.9)	0.779	0.342
Arginine	68.9 (55.7-85.8)	66.5 (55.9-81.6)	0.008	0.988
Asparagine	77.8 (67.7-85.0)	78.5 (64.8-91.6)	0.032	0.872
Aspartate	13.9 (10.5-17.9)	14.9 (11.1-20.3)	2.335	0.103
Glutamine	433.4 (379.5-534.4)	389 (312.8-474.7)	5.935	0.024[a]
Glutamate	39.3 (27.2-46.5)	42.0 (34.2-53.5)	4.130	0.039[a]
Glycine	418.4 (374.7-455.2)	396.7 (340.5-469.7)	0.181	0.724
Proline	316.3 (219.0-418.1)	254.6 (207.2-319.8)	3.690	0.045[a]
Serine	83.2 (68.7-92.8)	82.5 (66.1-97.0)	0.034	0.934
Taurine	64.5 (50.6-82.1)	66.4 (55.6-82.7)	0.709	0.416
Tyrosine	82.9 (72.6-98.3)	85.5 (75.7-101.0)	0.090	0.718
Citrulline	54.1 (42.1-70.1)	50.9 (42.4-64.9)	0.548	0.541
Ornithine	58.2 (46.5-71.1)	60.4 (45.8-83.0)	0.068	0.882
Phosphoserine	54.7 (26.9-89.8)	50.3 (31.5-66.1)	0.502	0.435
Hydroxyproline	11.4 (1.6-18.7)	16.2 (11.3-20.8)	4.752	0.018[a]

[a]P<0.05; [b]Data are presented as the median (inter-quartile range). ADHD, attention deficit/hyperactivity disorder.

Table III. Multivariate linear regression analysis of the association between serum amino acid levels (independent predictor) and attention deficit/hyperactivity disorder Rating Scale-IV scores (dependent variable).

	Model 1		Model 2	
Amino acid	β	P-value	β	P-value
Ala	0.140	0.460	0.050	0.724
Asp	-0.009	0.965	-0.077	0.596
Gln	-0.353	0.022[a]	-0.370	0.002[b]
Glu	0.258	0.206	0.350	0.029[a]
His	-0.150	0.218	-0.057	0.583
Hypro	0.227	0.111	0.211	0.065
Lys	-0.527	0.027[a]	-0.339	0.021[a]
Orn	0.281	0.087	0.161	0.248
Pro	-0.051	0.643	-0.082	0.391
Thr	0.327	0.081	0.169	0.214
Multiple R	0.576	0.518		
Multiple R^2	0.332	0.268		
Adjusted R^2	0.100	0.170		
P for the model	0.116	0.004[b]		

[a]P<0.05, [b]P<0.01. Data are expressed as the regression coefficient (β) and the respective P-value.

Gln and Lys levels were found to be inversely associated with total ADHD-RS scores. The overall model trended towards statistically significant (P=0.072), accounting for only 12% of total ADHD-RS scores. In a model incorporating amino acids considered to be significantly and almost significantly associated with ADHD scores (Model 2), serum Gln and Lys remained significantly associated with ADHD, whereas circulating Glu levels appeared to be positively associated with total ADHD-RS scores. Serum Hypro levels were almost significantly associated with ADHD scores. Although the predictive value of Model 2 was significant, it accounted for only 17% of score variability. Neither age nor sex were associated significantly with ADHD in both regression models.

Discussion

The results of the present study demonstrated that children with ADHD were characterized by distinct amino acid profiles compared with the controls, indicative of predominant alteration in Glu, Pro and Lys metabolism. Significant group differences in Glu/Gln ratio may be indicative of altered neurotransmission in children with ADHD, whereas high Hypro levels and a high Hypro/Pro ratio may be considered as a marker of collagen catabolism and connective tissue pathology.

Existing data demonstrate that alterations in glutamatergic signaling may contribute significantly to ADHD pathogenesis (6). Specifically, it has been demonstrated that ADHD patients are characterized by significantly lower Gln plus Glu levels in basal ganglia (18), including in the striatum (19). An increase in anterior cingulate cortex Glu content was also associated with hyperactivity and impulsivity in adult ADHD patients (20). Pertinent genome-wide analyses for ADHD risk genes revealed altered expression profiles of genes associated with glutamatergic neurotransmission (21). Correspondingly, altered AMPAR-mediated transmission in pyramidal neurons of the prefrontal cortex was also found to be associated with ADHD in an experimental rat model (22).

In view of the role of Hypro as a marker of connective tissue pathology (23), the earlier proposed association between

ADHD and joint hypermobility (24) may underlie the observed increase in plasma Hypro levels in children with ADHD. Particularly, joint hypermobility was found to be >2-fold more prevalent in ADHD subjects compared with the reference population (25). Moreover, connective tissue disorders are also closely associated with other neurodevelopmental disorders (26). These data also corroborate our earlier findings on increased Hypro levels in autism spectrum disorder (27) and cerebral palsy (28). In addition, in ADHD subjects, Pro levels were found to be reduced with a decreased Pro/Hypro ratio, and levels were found to be inversely associated with S100B and positively related to IL-10 levels (29), indicative of the potential contribution of dysregulated Pro metabolism in this neurodevelopmental disorder.

The results of serum amino acid profiling also demonstrated group differences in serum His, Asp and Lys levels between ADHD patients and neurotypical controls. Although an essential role of His in brain physiology has been demonstrated (30), data on the relationship between ADHD and His metabolism are insufficient. His supplementation was shown to reduce fatigue and improve mental performance in subjects with high fatigue and sleep disruption scores (31). In turn, experimental data demonstrated that a His-deficient diet resulted in formation of anxiety-like behaviors in mice through reduction of brain histamine levels (32). In turn, increased serum Asp levels in patients with ADHD generally correspond with an earlier observed higher Asp intake in ADHD patients (33).

It is noteworthy that the present study revealed a significant association between lower serum Lys levels and ADHD. Despite the role of Lys metabolism in brain physiology (34), earlier data on the involvement of Lys in ADHD pathophysiology are lacking. Nonetheless, Lys supplementation has been used in ADHD management (35), based on observations that Lys as well as Arg treatments reduce anxiety in stressed adults characterized by high cortisol levels (36).

In contrast to earlier reports (11,12), significant group differences in tyrosine, phenylalanine or Trp levels were not observed in the present study, in agreement with a study by Bergwerff *et al* (13) who did not reveal any significant group differences in serum and urinary levels of the studied amino acids (13).

The present study also has several limitations. First, this was a cross-sectional study involving a relatively small number of patients, and studying larger cohorts of both cases and controls may improve the statistical power of the results. Second, follow-up design of the study with evaluation of ADHD incidence and severity would be beneficial to provide an insight into the causal relationships between ADHD and the observed alterations in amino acid metabolism. Third, although the results demonstrate the potential alterations of amino acid-derived neurotransmitters in patients with ADHD, serum levels do not necessarily reflect brain levels of amino acids and their derivatives. Therefore, evaluation of cerebrospinal fluid amino acid levels or the use of techniques for direct brain metabolite assessment, such as proton magnetic resonance spectroscopy, would assist in highlighting ADHD-associated alterations in metabolism of brain amino acids and neurotransmitters.

In conclusion, the results of the present study demonstrated significant alterations in the serum amino acid profile of children with ADHD. The observed alterations of Pro/Hypro and Gln/Glu levels and their ratios may be associated with the coexisting connective tissue pathology and alterations of glutamatergic neurotransmission in ADHD, respectively. Altered circulating levels of His, Lys and Asp may also be implicated in the pathogenesis of ADHD. However, further *in vitro* and *in vivo* studies are required to investigate the specific underlying mechanisms linking amino acid metabolism with ADHD pathogenesis.

Acknowledgements

Not applicable.

Funding

The present study was supported by funding from Russian Foundation for Basic Research (RFBR) (grant no. 19-013-00528).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AVS, MGS and AAT conceived the study. AVS, AAT, AT, DAS and MA designed the study. AVS, AAT, AAS, ALM, IPZ, YNL and MGS performed the experiments. ALM, IPZ, AVS, AT and DAS analyzed the data. ALM, AAT, AAS, IPZ, YNL, AVS, MGS, AT, DAS and MA wrote and edited the manuscript. All authors have read and approved the final manuscript. AVS, MGS and AAT confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was performed in accordance with the ethical principles of the Declaration of Helsinki and its amendments (2013). The protocol of the present study was considered and approved by the Local Ethics Committee (Yaroslavl State University, Yaroslavl, Russia). Parents or legal representatives signed an informed consent form for participation of their children in the present study prior to investigation. Examination and blood sample collection was performed only in the presence of the parents/guardians.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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