Sulfur amino acid-free diet results in increased glutamate in human midbrain: a pilot magnetic resonance spectroscopic study

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Accessed January 18, 2020 9:11 PM EST

Sulfur amino acid-free diet results in increased glutamate in human midbrain: A pilot magnetic resonance spectroscopy study

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Abstract

Objective—This pilot study was designed to determine if metabolic effects in different brain regions (left and right parietal lobes, midbrain) due to 3 days of food consumption without methionine or cysteine could be detected by proton magnetic resonance spectroscopy (MRS).

Research Methods & Procedures—Healthy individuals aged 18-36 y (n=8) were studied by MRS after receiving diet with adequate sulfur amino acids (SAA) or with zero SAA for 3 days. Pulse sequences were used to selectively measure glutathione (GSH) and linear combination modeling (LCM) of spectra was used to measure other high abundance brain metabolites, and expressed relative to creatine (Cr).

Results—Although dietary SAA are required to maintain glutathione (GSH), the 3-d SAA insufficiency resulted in no significant change in GSH/Cr in the three brain regions. Principal component analysis of 16 metabolites measured by LCM showed that the metabolic pattern in the midbrain, but not the parietal lobes, was distinguished according to the dietary SAA. Multivariate statistical analysis showed that the major discriminating factors were signals of glutamate/Cr, (glutamate+glutamine)/Cr, and myo-inositiol/Cr. Correlation analyses between midbrain metabolites and GSH-related metabolites in plasma showed that midbrain glutamate/Cr had an inverse correlation with plasma cystine.

Conclusion—The data show that MRS is a non-invasive tool suitable for nutritional assessment and suggest that nutritional imbalance caused by 3-d of sulfur amino acid-free food more selectively affects midbrain than the parietal lobes.

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Keywords

Brain metabolism; oxidative stress; amino acid metabolism; nutritional assessment; protein malnutrition; glutathione

Introduction

Short-term nutritional insufficiencies occur with many medical and surgical conditions, yet the specific contributions to outcomes of such insufficiencies are poorly understood and difficult to routinely assess [1-4]. Several factors contribute to the severity of nutrient insufficiency, including the nature of medical or surgical conditions, diet intake history, and the duration of inadequate food intake [5-8]. Thus, assessment of nutrient needs under challenging medical circumstances is essential to guide nutrition support therapy [9-11].

The body has no mechanism to store amino acids with the consequence that as little as 1 day of inadequate intake can result in muscle degradation to maintain amino acid supply for new protein synthesis. Cysteine (Cys) is an amino acid which can be insufficient in the short term due its use as a precursor for the antioxidant glutathione (GSH) [12-15]. In clinical conditions requiring parenteral nutrition, Cys is omitted because of instability in solution, and cystine (CySS), another source of Cys through interconversion, is omitted because of poor solubility [16, 17]. Although Cys can be synthesized from methionine, this can be limited by hepatic insufficiency, which is common in many catabolic conditions. The need for Cys to maintain GSH can be exacerbated in some individuals due to low GSH associated with disease states, e.g., HIV, hepatitis, protein-energy malnutrition, chronic kidney failure, Alzheimer's and Parkinson's disease [18-22]. Compared with other organs, the brain may be relatively vulnerable because the brain requires GSH to protect against a high oxidative metabolism [23, 24]. A decrease in GSH is associated with increased sensitivity of cells to apoptosis and with decreases in recovery and repair activities [25-27]. Thus, GSH could serve as a quantifiable indicator of the need for antioxidant support [15, 28, 29].

Proton magnetic resonance spectroscopy (^{1}H-MRS) uses magnetic resonance imaging (MRI) instrumentation to quantify metabolites in tissues based upon the magnetic properties of protons in a high field magnet [30-32]. In MRS applications, an organ is imaged to position a region for analysis and then a spectrum is recorded for a defined 3-dimensional volume. The spectrum is complex, consisting of overlapping signals due to multiple chemicals, and limited sensitivity allows detection of only high abundance chemicals. Previous studies have provided pulse sequences which allow specific measurement of GSH in the human brain [33-35]. Because there are difficulties with absolute quantification, measurements are expressed relative to other high abundance metabolites, such as creatine (Cr). Additionally, software is available which uses computer-based modeling of MRS spectra of known metabolites to estimate quantities of other high-abundance metabolites, including amino acids, carbohydrates, lipids, and related metabolites, e.g., glutamine, glutamate (Glu), N-acetylaspartate, N-acetylaspartylglutamate, GABA, glucose, myo-inositol, glycerophosphorylcholine and phosphocreatine [36].

The purpose of this pilot study was to determine whether MRS could detect a decrease in GSH in human brain regions (left and right parietal lobes, midbrain) following a 3-d SAA insufficiency in healthy individuals. We hypothesized that SAA insufficiency would be associated with a decrease in the ratio of GSH to creatine (Cr) in three brain regions including left and right parietal and midbrain. A secondary purpose was to determine whether glutamate, or other detectable metabolites, changed in association with the SAA intake. Our data show that an insufficiency of SAA for 3 days did not result in a significant

change in GSH/Cr in either region. Principal component analysis (PCA) of metabolites detected by MRS showed that SAA insufficiency altered metabolite patterns in midbrain but not those of left or right parietal lobes.

Materials and Methods

Human subjects

This study was reviewed and approved by the Emory Investigational Review Board (IRB#: 1098-2003); the study represented ancillary measurements to a study of SAA-insufficiency effects on plasma thiol/disulfide redox potential [37]. Because this was a pilot study, there were no power calculations. Details of the 13-day study of plasma Cys/CySS and GSH/ GSSG redox states are available [37]. Briefly, the study was performed in 8 healthy young adults. After informed consent, subjects were screened based upon medical histories, physical examinations, standard blood chemistry, hematology, urinalysis and a serum pregnancy test (females). Eligibility to participate was established by the absence of evidence of acute or chronic illness, no current smoking history, and a BMI less than 27 kg/ m^2. Subjects taking antioxidants, nutrient supplements (with the exception of once-daily multivitamin-mineral supplements) or acetaminophen were asked to discontinue these two weeks before the onset of the studies.

The overall design included a 3-d equilibration on normal food approximating the RDA for SAA (12.2 mg/kg Met plus 6.6 mg/kg Cys), 5 d of SAA-free food (SAA-free) and 4.5 d of SAA-containing food (37.3 mg/kg Met plus 18.7 mg/kg Cys). The SAA intake during the latter repletion phase was 56 mg/kg SAA per day with a distribution of Met:Cys of 2:1, selected to approximate the mean intake in the American diet as determined by NHANES III. On the first and last day of each SAA-free or SAA- containing period, blood was drawn hourly. Thus, to avoid interference with these collections, the study was designed to have MRS performed on Day 4 of each study period, thereby allowing paired analysis of brain GSH within individuals. However, difficulties in scheduling (volunteer schedule/research ward availability/MRI instrumentation availability) and instrument failure, resulted in inability to obtain the desired paired MRS data for 3 out of the first 4 subjects enrolled. Consequently, the protocol was modified to collect MRS for adequate SAA intake on either the last day of equilibration (two individuals) or the 4th day of SAA repletion (3 individuals), and these SAA-replete conditions were compared to the SAA-insufficient condition on the 4th day of the SAA-free period. For one of these individuals, useful data could not be obtained for the midbrain region because of magnetic field inhomogeneity in the volume of interest (VOI).

Acquisition of MRS Spectra

Fasting plasma was collected in the GCRC at 0830 for determination of plasma GSH, GSSG, cysteine and cystine, and the subject was then transported to the imaging facility. The time between blood sampling and ^{1}H-MRS measurement could not be precisely controlled but was within 2 h for all subjects. MRS data were acquired using a 3T Siemens Magnetom Trio system (Siemens Medical Solutions, Malvern, PA) equipped with a Sonata gradient set capable of 40 mT/m with a maximum rise time of 200 μs. Subjects were positioned supine inside the magnet. A transverse electromagnetic (TEM) head coil was used for excitation and detection [35]. After a localizing scan, a high-resolution T1-weighted 3D anatomical image was acquired with the MPRAGE sequence (176 sagittal slices, TR/ TE=2600/3.93msec, flip angle=8°) (Fig 1). Shimming on the volume of interest (VOI) of all first- and second-order spectra was achieved by the automatic shimming technique on the scanner. GSH was detected using double quantum editing methods (CHESS) as described before [35] with the following parameters: TR= 1.5s, 2048 complex data points and a 2000

Hz bandwidth. After 4 dummy scans, 512 *in vivo* scans were collected and averaged. GSH/ N-acetylaspartate (NAA) ratio was determined using co-edited NAA peaks through a custom Matlab program [35]. For the same voxel, ^{1}H-MRS spectra were acquired using the point-resolved spectroscopy localization sequence (PRESS) with TE = 30 ms, TR = 3 s, TM=20 s, and 64 averages. The fitted T2* values were used as an indication of the quality of the *in vivo* spectra, and spectra with less than 50 ms in T2* were excluded because of poor spectral quality.

Linear Combination Modeling (LCM)

LCM is a method to quantify metabolites by fitting the spectra from the PRESS sequence with a linear combination of metabolite spectra [36]. A single voxel in proton magnetic resonance spectroscopy provides a rapid biochemical profile of a localized VOI. In this study, the concentrations of metabolites were standardized and expressed in terms of the ratio of Cr to minimize errors arising from changes in tissue volume or variation in magnetic field homogeneity [38, 39]. The contribution of individual metabolites to the *in vivo* spectrum was quantified using LCM; the following metabolites were included in the basis set: aspartate (Asp), glycerophosphorylcholine (GPC), phosphorylcholine (PCho), creatine (Cr), phosphocreatine (PCr), GABA, glucose (Glc), glutamine (Gln), glutamate (Glu), myo-inositol (myo-Ins), lactate (Lac), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG), phosphorylethanolamine (PE), scyllo-inositol (scyllo-Ins), taurine (Tau), and macromolecules (MM).

Statistical Analysis and Principal Component Analysis (PCA)

Statistical comparisons for GSH/Cr during SAA-free and SAA-containing diet periods were performed for each parameter with a pairwise *t*-test for each region of the brain. P-values of < 0.05 were considered significant. The data are expressed as means ± SEM.

To examine data for metabolic patterns associated with consumption of SAA-free food, data reduction by PCA was performed using Pirouette software (Infometrix, Bothell, WA) on the metabolite area ratios related to creatine. Three-dimensional PCA score plots and loading plots were used to visualize relational patterns and discriminatory factors to classify the groups [40]. The first three principal components accounted for 90% of the total variation and were used in 3D score plots to visualize differences between spectra. Loading plots were used to identify the individual metabolites contributing to the score plot [41, 42].

Results

Study subject characteristics

Eight subjects ages 18-36 y were studied (5 males, 3 females; 3 whites, 4 blacks, and 1 Asian), mean age (± SEM) 23.6 ± 2.7 y and body mass index 22.5 ± 1.8 kg/m^2. Subjects reported no acute or chronic illnesses and none were taking regular prescription medications.

The relative quantification of GSH/Cr in three brain regions

GSH measurements were obtained in 3 brain regions in a total of 8 subjects with SAA insufficiency and 8 subjects with adequate intake (Fig 2). Usable data were obtained for the left and right parietal regions for all 8 subjects, but data for the midbrain region of 3 subjects were excluded from analysis because of poor spectral quality. Unpaired ANOVA showed no significant differences in GSH according to region. Mean values for combined left and right parietal lobes for insufficient SAA intake (GSH:Cr, 0.24 ± 0.02; n = 16) and adequate SAA intake (GSH:Cr, 0.26 ± 0.02, n = 16), were not significantly different. Calibration relative to external standard solutions containing GSH showed that these concentrations were approximately 0.91 ± 0.16 for left parietal and 0.89 ± 0.16 mM for right parietal,

respectively. For 6 subjects, data were obtained on both days, so paired analysis was performed on these subjects; no significant differences were observed. Thus, the primary outcome of this pilot study provided no evidence that short-term (3-d) intake of food with inadequate SAA content has a detectable effect on brain GSH in left parietal, right parietal or midbrain regions. Comparisons of GSH:Cr content between brain regions also showed no significant difference (p<0.09).

While the direct outcomes of these measurements were negative, the results provide useful information for future experimental design. Based upon the 20% SD ($\alpha=0.05$, $1-\beta=0.8$) of these analyses, a sample size of at least 33 subjects would be needed to determine whether the level of GSH changed by 10% in the midbrain region. To observe significance (20% SD, $\alpha=0.05$, $1-\beta=0.8$) at a 5% change in the level of GSH, power analysis indicates that 128 subjects would be required. Thus, the data show that the sensitivity of the MRS assay with current technology limits the ability to detect potentially important changes in GSH in specific brain region without study of a relatively large number of individuals.

Quantitative analysis of 16 metabolite/Cr ratios in brain using Linear Combination Modeling (LCM) of ^{1}H-MRS spectra—^{1}H-MRS spectra of the different brain regions with adequate SAA or devoid of SAA for 3 d were analyzed using the LCM computer program (Table 1). We conducted 2-way ANOVA and post-hoc analyses to test for differences in concentration of each metabolite between brain regions and possible effects of SAA insufficiency on metabolite concentrations. The results showed that the metabolite concentrations were not uniform in different parts of the brain. In particular, the concentrations of glutamine (Gln), myo-inositol, glycerophosphorylcholine (GPC), and GPC + phosphorylcholine (PCho) in the midbrain were significantly higher than in the left and right parietal regions. The analysis also revealed that the SAA insufficiency significantly affected levels of Glu, and Glu+Gln (p < 0.05).

Principal Components Analysis (PCA) on metabolite/Cr ratios measured by LCM—To further test for changes in the pattern of metabolites associated with insufficient SAA intake, PCA was performed on metabolite/Cr ratios in each of the brain regions. The PCA showed that the first three PCs represented close to 90% of the total variation in data for each of the brain regions. Score plots of the first 3 Principal Components for the left and right parietal lobes did not show any separation related to SAA content of the diet (Fig 3). In contrast, the score plot of the PCA for midbrain metabolites showed distinct patterns according to the whether SAA was available in the diet (Fig 3).

To provide information on the major metabolite/Cr ratios which contributed to the discrimination between the midbrain metabolic profiles with and without SAA, a loading plot was used in which the orientation of PCs in the loading plot were aligned with the same orientation as the score plot (Fig 4). The data indicated that Glu, Gln, and myo-inositol were major contributors to the difference between SAA-free and SAA-containing diet groups in the midbrain region. To further test this relationship, paired *t*-tests were performed on each metabolic ratio with and without SAA in three different regions (Table 1). The concentrations of Glu in the midbrain and in the left parietal lobe increased significantly in the SAA-insufficient period. Myo-inositol also significantly increased in the midbrain in the SAA-insufficient period (Table 1).

Taurine, another metabolite of Cys, can be detected by MRS of brain but has not previously been reported in the LCM analysis in the midbrain. To determine whether taurine changed in association with SAA insufficiency, spectra were individually analyzed for the taurine signal. A signal for taurine could be distinguished for the left and right parietal regions but not in midbrain (Table 1). The concentration of taurine was not significantly different

between the groups with and without SAA in the left and right parietal regions. Consequently, the combination of analyses showed that 3 d of SAA-free diet had no detectable effect on GSH, taurine or most other metabolites detected in the brain by ^{1}H-MRS. In contrast, significant effects were detected on Glu and myo-inositol in the midbrain region.

Correlation analysis of brain metabolites and plasma GSH, GSSG, Cys and CySS

Plasma GSH, Cys and CySS undergo diurnal variation with an apparent association with dietary intake of food [43]. To determine whether brain metabolites correlated with plasma aminothiols, regression analyses were performed for each brain metabolite in Table 1 with plasma GSH, GSSG, Cys and CySS concentrations measured in plasma taken within 2 h of the ^{1}H-MRS spectra. The results showed that midbrain Glu:Cr was strongly and inversely correlated with plasma cystine concentration (Fig 5). The correlation coefficient (R^2) was 0.563 (p-value = 0.032). Consequently, the data provide evidence that consumption of a diet free of SAA for 3 d results in an increase in the midbrain Glu, which is correlated to decrease in plasma CySS.

Discussion

Noninvasive methods for determining the amount of GSH and other metabolites in the brain could be useful for studies of neurodegenerative motor diseases, such as Parkinson's disease, in which changes in GSH are thought to be an important contributor to oxidative stress and possibly pathogenesis. The present pilot study indicates that 3 d without dietary SAA intake in young healthy adults does not cause a major decrease in brain GSH/Cr as measured by ^{1}H-MRS. These results provide data for power calculations for future ^{1}H-MRS studies to determine whether nutritional interventions, such as involving N-acetylcysteine (NAC) or other GSH precursor, affect midbrain or parietal GSH. The present data show that detection of relevant changes in GSH in the midbrain with current technology will require relatively large numbers of subjects. In addition, current MRS technology does not allow measurement of metabolic changes exclusively in the substantia nigra because of tissue inhomogeneity and poor signal to noise with such a small voxel size.

The MRS spectrum represents radiofrequency signals from the proton nuclei of the different metabolites in the region of interest [30, 31, 35, 44]. Specific metabolites always appear at the same frequencies, expressed as parts per million on the horizontal axis of the graph using the water as an internal standard. MRS with LCM [36] allows one to estimate the quantities of individual metabolites in the brain using a least-squares optimization algorithm. LCM can be used to measure concentration of metabolites, which is proportional to the area under the peak, relative to other metabolites or to external standards. In this study, creatine (Cr) was used as a reference because previous studies showed that the creatine peak is essentially constant due to high metabolic demands imposed by brain cells [45]. The calculation of GSH concentration relative to external standards supports the validity of this use. In addition, expression of individual values as ratios to NAA, another metabolite which has been used as a reference, produced similar results (data not shown).

The metabolite data suggest that application of LCM to MRS spectra of the brain can be useful for non-invasive nutritional assessment or measurement of metabolic effects of therapeutic interventions. In the present study, LCM provided useful measurement of 16 metabolites in 3 brain regions. Although the targeted analysis of GSH did not show effects of the dietary manipulation, the application of PCA to all metabolites showed an unanticipated discriminatory pattern in the midbrain associated with SAA insufficiency. Importantly, the results showed that Glu:Cr and myo-inositol:Cr increased in midbrain in

response to SAA insufficiency. No other significant effects were observed except that Glu:Cr was also increased in the left parietal region.

Multivariate statistical analysis further showed that the change in midbrain Glu was inversely associated with plasma CySS. While this observation did not result from a test of a pre-stated hypothesis, the results are consistent with known transport characteristics of glia, where the x_c^- system exchanges CySS for Glu to protect neurons from excessive extracellular Glu [46-48]. The data suggest that under conditions of high plasma CySS, the extracellular CySS concentration may also be high in the brain and alter the glial function in Glu regulation. Such a change could contribute to altered sensitivity of neurons to glutamate toxicity or altered availability of intracellular Glu as a mitochondrial respiratory substrate in glia. Additional studies are needed to explore the mechanisms and biological significance of these changes because the results could indicate either a general response to imbalanced amino acid intake or a specific effect related to SAA insufficiency. None-the-less, the results show that discovery-based metabolic profiling using MRS can provide a novel means for nutritional assessment.

In summary, the present study shows that ^{1}H-MRS with LCM can be used to measure metabolic changes in human brain regions associated with nutritional interventions, especially providing the ability to detect changes in response to an amino acid imbalance created by a diet with insufficient SAA content. Because the required MRI instrumentation is widely available, ^{1}H-MRS could provide a novel approach for *in vivo* clinical nutritional assessment.

Acknowledgments

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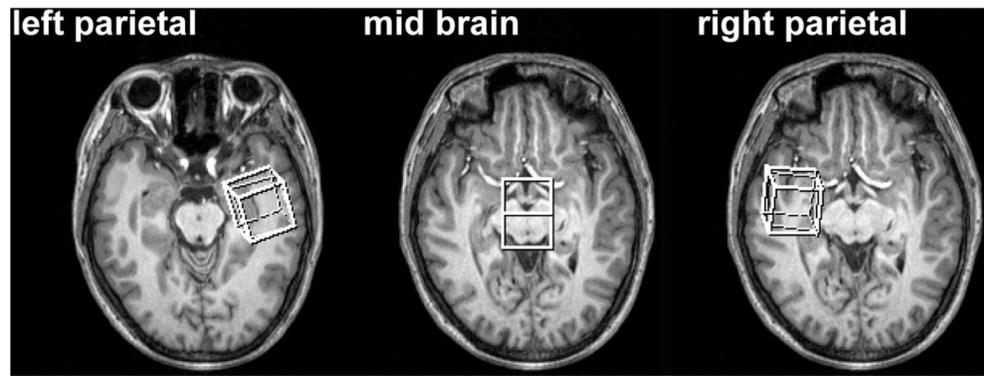
Fig 1. Human brain regions used for Magnetic Resonance Spectroscopy
The anatomical scan protocol had a 3D FOV = $256\times224\times176$ mm^3 and a 3D matrix = $256\times224\times176$ resulting in an isotropic resolution of 1 mm. The volumes of interest (VOI, $30\times30\times30$ mm) for spectroscopic studies in the left parietal lobe, right parietal lobe and midbrain were selected using high-resolution anatomic images as illustrated by the cubes.
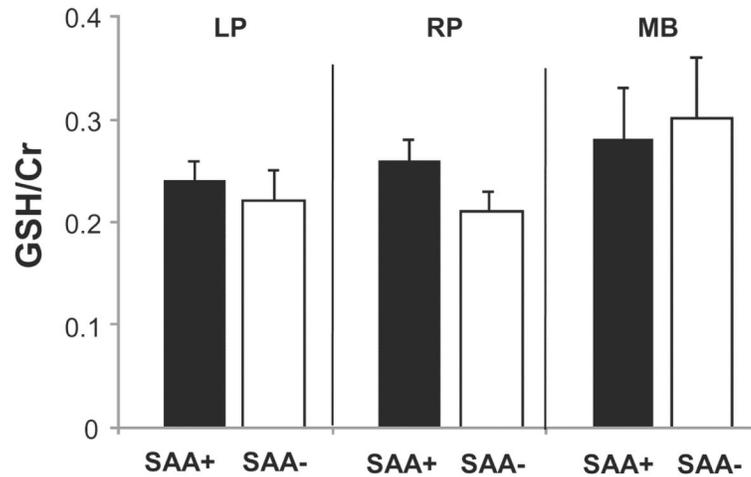
Fig 2. Effect of dietary intake of SAA on the relative abundance of GSH in the left parietal, right parietal and midbrain regions of humans

Data are expressed as a ratio of the creatine (Cr) signal following 3 days with and without dietary SAA. No significant differences were observed. At the level of sensitivity obtained, a change of less than 25% could not have been detected. The number of subjects for this study of left parietal was 8. The number of subjects for right parietal was 8. The number of subjects for midbrain was 5.
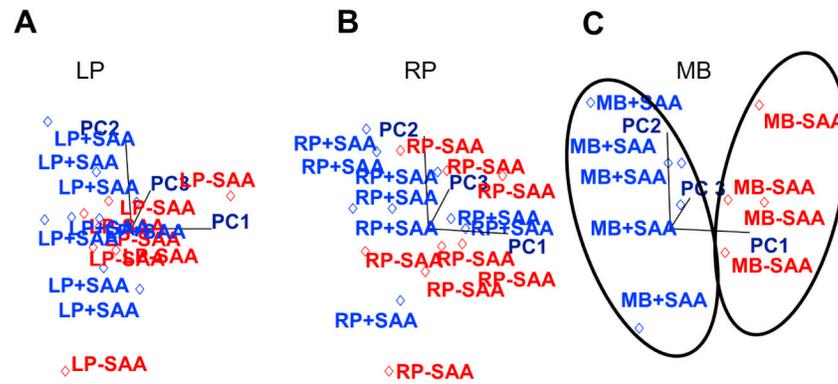
Fig 3. Unsupervised Principal Component Analysis (PCA) of the effects of dietary sulfur amino acid (SAA) intake on abundant metabolites in left parietal, right parietal and midbrain regions of humans

Subjects were equilibrated for 3 days with a diet containing an adequate SAA intake (+SAA, blue labels) or isoenergetic, isonitrogenous food without SAA (-SAA, red labels). Magnetic resonance spectroscopy and Linear Combination Modeling was used to quantify 16 metabolites in the left parietal (LP) (left), right parietal (RP) (middle) and midbrain regions (MB) (right) as provided in Table 1. Data reduction by unsupervised PCA was performed using Pirouette software with color labels added to easily visualize separation. No differences in metabolic patterns were observed for LP or RP regions of the brain; however, separation due to metabolic profiles in the MB region was readily apparent.
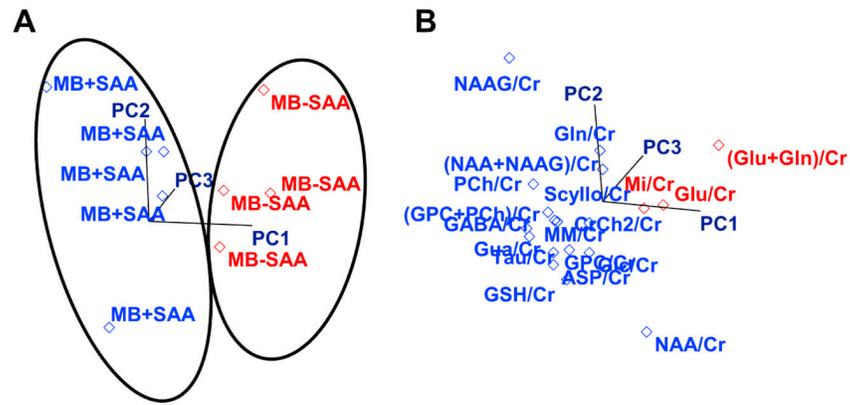
Fig 4. Principal Component Analysis (PCA) of effects of dietary sulfur amino acid intake on human midbrain metabolites
A. PCA score plot. Red represents +SAA; blue represents -SAA. B. PCA loading plot. The far right blue diamonds represents the major discriminatory factors to classify the groups, including mI/Cr, Glu/Cr and (Glu+Gln)/Cr.
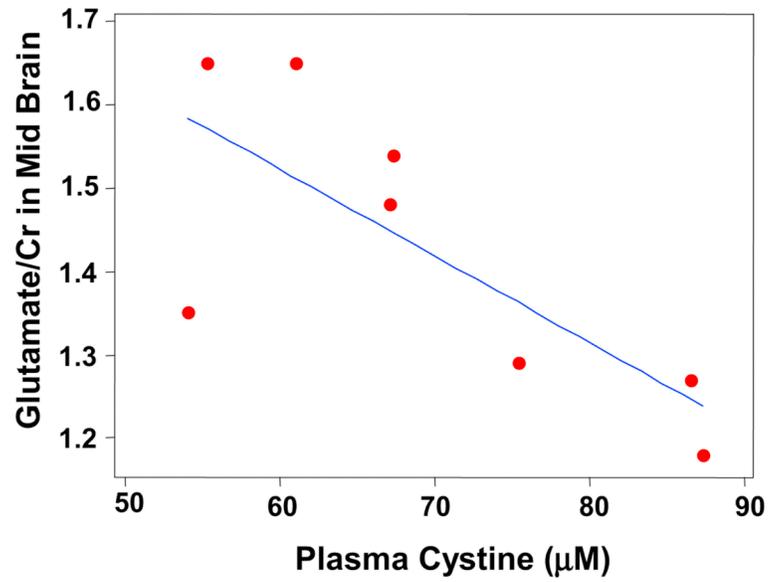
FIG. 5. Regression plot between plasma cystine and glutamate-to-Cr ratio in the human midbrain
Data are for individuals summarized in Table 1; plasma cystine samples were obtained within 2 h of MRS measurements.

Table 1

The effect of dietary sulfur amino acids (SAA) on metabolite:creatine ratios in the left parietal (LP), right parietal (RP) and midbrain (MB) regions

	LP +SAA	LP -SAA	RP +SAA	RP -SAA	MB +SAA	MB -SAA
Glutamine/Cr	0.41±0.10	0.60±0.09	0.31±0.09	0.49±0.07	0.75±0.17	0.86±0.09
Glutamate/Cr	1.21±0.08	1.45±0.10 **	1.31±0.07	1.39±0.09	1.28±0.04	1.58±0.04 **
Asparate/Cr	0.13±0.04	0.16±0.06	0.15±0.04	0.08±0.03	0.14±0.08	0.18±0.07
GABA/Cr	0.24±0.06	0.19±0.05	0.20±0.02	0.24±0.03	0.36±0.06	0.23±0.03
Glucose/Cr	0.29±0.05	0.19±0.06	0.24±0.03	0.29±0.03	0.22±0.07	0.32±0.05
GPC/Cr	0.19±0.01	0.12±0.03	0.20±0.02	0.21±0.01	0.27±0.05	0.27±0.01
myo-Inositol/Cr	0.80±0.06	0.70±0.08	0.79±0.04	0.78±0.04	0.64±0.04	0.84±0.02 *
Taurine/Cr	0.13±0.04	0.18±0.05	0.10±0.02	0.07±0.03	ND	ND
NAA/Cr	1.33±0.05	1.31±0.05	1.35±0.03	1.29±0.04	1.25±0.04	1.43±0.06
NAAG/Cr	0.15±0.02	0.14±0.04	0.14±0.03	0.19±0.04	0.17±0.09	0.11±0.07
GuanidoAc/Cr	0.16±0.04	0.17±0.05	0.18±0.04	0.08±0.02	0.10±0.05	0.03±0.02
(GPC+PCho)/Cr	0.20±0.01	0.20±0.01	0.22±0.01	0.22±0.01	0.27±0.01	0.27±0.01
(NAA+NAAG)/Cr	1.49±0.06	1.45±0.04	1.49±0.04	1.48±0.08	1.42±0.05	1.54±0.06
(Gln + Glu)/Cr	1.83±0.11	2.05±0.17	1.62±0.10	1.88±0.13	2.03±0.14	2.44±0.10
-CrCh₂/Cr	0.07±0.02	0.07±0.03	0.12±0.03	0.07±0.03	0.08±0.04	0.15±0.035

Abbreviations: GPC, glycerophosphorylcholine; PCho, phosphorylcholine; Cr, creatine; PCr, phosphocreatine; GABA, gamma-aminobutyric acid; Glc, glucose; Gln, glutamine; Glu, glutamate; GuanidoAc, guanidoacetate; myo-Ins, myo-inositol; NAA, N-acetyl-aspartate and NAAG (N-acetyl-aspartyl-glutamate). Data were analyzed by 2-way ANOVA for brain region and effects of SAA in food. Analysis showed significant differences associated with region as follows: Two-way ANOVA analysis was used to compare the effects of region and SAA intake. Results showed effects of both region and SAA intake. The concentrations of Gln, myo-inositol, GPC and GPC + PCho in the midbrain were significantly different from the left and right parietal regions (p<0.05). The SAA intake affected significantly levels of Glu, and Glu+Gln (p < 0.05).

Additional pair-wise t-tests were performed in response to PCA analysis which indicated that Glu and myo-inositol contributed to differences in midbrain region between SAA intake levels. Significant results from pairwise t-tests are identified in the table as follows:

*
Significantly different at p < 0.05;

**
Significantly different at p < 0.01.