High-Resolution Magic-Angle Spinning-1H-NMR Spectroscopy-Based Metabolic Profiling of Hippocampal Tissue in Rats with Depression-Like Symptoms

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Received September 30, 2016; accepted February 27, 2017; advance publication released online March 4, 2017

Depressive disorders cause large socioeconomic effects influencing not only the patients themselves but also their family and broader community as well. To better understand the physiologic factors underlying depression, in this study, we performed metabolomics analysis, an omics technique that comprehensively analyzes small molecule metabolites in biological samples. Specifically, we utilized high-resolution magic-angle spinning-1H-NMR (HRMAS-1H-NMR) spectroscopy to comprehensively analyze the changes in metabolites in the hippocampal tissue of rats exposed to chronic stress (CS) via multi-step principal component analysis (multi-step PCA). The rats subjected to CS exhibited obvious depression-like behaviors. High correlations were observed between the first principal component (PC1) score in the score plot obtained using multi-step PCA and measurements from depression-like behavioral testing (body weight, sucrose preference test, and open field test). Alanine, glutamate, glutamine, and aspartate levels in the hippocampal tissue were significantly lower, whereas N-acetylaspartate, myo-inositol, and creatine were significantly higher in the CS group compared to the control (non-CS) group. As alanine, glutamate, and glutamine are known to be involved in energy metabolism, especially in the tricarboxylic acid cycle, chronic exogenous stress may have induced abnormalities in energy metabolism in the brains of the rats. The results suggest that N-acetylaspartate and creatine levels may have increased in order to complement the loss of energy-producing activity resulting from the development of the depression-like disorder. Multi-step PCA therefore allowed an exploration of the degree of depression-like symptoms as represented by changes in intrinsic metabolites.

Key words depressive disorder; multi-step principal component analysis; brain tissue; metabolomics; high-resolution magic-angle spinning-1H-NMR; N-acetylaspartate

According to a survey of the WHO in 2012, over 350 million individuals exhibit depressive disorder, representing approximately 5% of the world population and over half of the approximately one million people who commit suicide every year. Accordingly, depressive disorders increasingly contribute to the Global Burden of Disease and are expected to be ranked as the primary contributor by 2030, overtaking ischemic heart disease. In Japan, the 2008 Patient Survey by the Ministry of Health, Labour and Welfare showed that there are over one million patients with mood disorder in Japan and that of these, approximately 700 000 have depressive disorder. Aside from the consequences of suicide, the development of depressive disorder results in a decrease in labor productivity owing to absenteeism, presenteeism, and non-employment, causing large socioeconomic effects. As the total cost of deaths and labor losses accounted to mental disorders such as depressive disorders reaches 7.2 trillion yen, the identification and establishment of measures related to ameliorating the socioeconomic effects of these diseases constitutes an urgent issue.

The diagnosis of depressive disorder and assessment of disease status are generally conducted based on voluntary self-examination and request of the patients themselves and by the observation of patient psychiatric symptoms and abnormal behaviors according to the Diagnostic and Statistical Manual of Mental Disorder-5 and the International Statistical Classification of Diseases and Related Health Problems-10. The disease is diagnosed by combining the results of a questionnaire completed by a patient and the interview conducted by a physician. Accordingly, the caveat exists that the diagnosis may vary depending on individual subjectivity.

An effective modern method to analyze disease pathophysiology is represented by metabolomics, an omics technique that comprehensively analyzes small molecule metabolites in biological samples. Within metabolomics analyses, NMR spectroscopy is frequently used as a tool that allows diagnosis support and a more comprehensive understanding of pathological conditions by identifying characteristic patterns in the metabolite changes in an organism caused by reaction or response to a disease. The usefulness of NMR-based metabolic profiling (NMR-MP) has been markedly demonstrated, for example, in the field of toxicology. Specifically, physiological changes in the body are assessed by performing a principal component analysis (PCA) of data obtained from NMR spectra and comprehensively analyzing the changes in metabolites from a distribution pattern. This method has been shown in several studies to allow the extraction of characteristics related to the physiological state in the body based on information regarding changes in metabolites assessed using measurements obtained from samples of homogenous biofluids such as urine, blood
and tissue extracts. In contrast, high-resolution magic-angle spinning (HRMAS)-$^1$H-NMR spectroscopy, which allows the collection of spectral data in a nearly non-destructive manner, is used in metabolomics analyses of inhomogeneous biological tissues such as cells$^{10}$ and biopsy samples.$^{11-13}$ Accordingly, we surmised that metabolic profiling (MP) via HRMAS-$^1$H-NMR spectroscopy might be utilized to identify a characteristic pattern in the expression of low molecule metabolites in the brain tissue of rats exhibiting depression-like symptoms consequent to the imposition of stress. In turn, this outcome would imply that the state of depressive disorder could be visualized by physicochemical measurements and that MP could be applied to grasp the pathologic development of the disease. Therefore, in this study we prepared an animal model that exhibits depression-like symptoms by imposing chronic stress to analyze whether the changes in metabolites in the brain tissue caused by stress might be visually assessed by NMR-MP.

MATERIALS AND METHODS

Animals We purchased 7-week-old male Wistar/ST rats from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan).

Basic Rearing Conditions Rats (three rats per cage) were reared in a steady environment (lights on: 07:00 to 19:00, temperature: 25±2°C, and relative humidity: 55±5%) with free access to food and water. The rats were fed Lab MR Stock (Nosan Corp., Kanagawa, Japan) and experiments were initiated following a habituation period of at least one week. In this study, we used a total of 12 rats (6 in the control group and 6 in the chronic stress (CS) group). All animal experiments were performed after approval by the animal experiment management campus committee at Josai University (approval number: H27024-2015/04/08).

Chronic Stress Procedure Chronic stress was imposed on the rats in the CS group; all six rats received the same stress stimulation (Fig. 1). The procedure was performed as described previously with modifications.$^{14-17}$ A combination of short- and long-term stressors was imposed each day. The short-term stressors included electrical shock (1.5 mA, 15 s duration and 60 s interval/single shock), forced swimming (for 5 min in a plastic tank (25 cm diameter) filled with water at 4°C at a depth of 24 cm), tail suspension (suspended for 1 min, 15 cm away from the ground, held using fingers 2 cm from the tip of the tail), and noise (85 dB, white noise). The long-term stressors included food and water deprivation (24 h), rearing in a tilted cage (45° for 21 h), constant light (no dark period; 12 h), rearing on wet bedding (24 h, 200 mL water per cage), and water deprivation (24 h, with an empty bottle for the last 1 h). The rats in the control group were not given the above stresses. After a habituation period, the rats were allocated to groups so that there would be no significant differences in body weight, after which they were reared individually.

Behavior Test Stress-induced depression-like symptoms in rats were assessed by performing standard behavioral pharmacological tests as previously reported.$^{18-20}$ “Body weight measurement,” “sucrose preference test,” and “open field test” were selected for assessing behaviors. We used a Student’s $t$-test to analyze the difference in the mean values of behavioral assessment between the control and CS groups. $p<0.05$ was considered statistically significant. We judged that a rat model of depressive disorder was successfully established when statistically significant differences were observed between the groups for all the mean values of behavioral assessment. The detailed behavioral assessments are described below.

Body Weight We measured body weight between 09:00 and 09:30 on days 0, 7, 14, and 21.

Sucrose Preference Test (SPT) We performed an SPT on days 1, 8, 15, and 22 according to a previous study.$^{18}$ First, in order to allow the rats to become used to the sucrose solution, two drinking bottles of 1% (w/v) sucrose were placed in the cage 72 h prior to the first SPT. At 24 h after the placement, one of the two drinking bottles
was changed to a bottle of pure water (Elix® UV, Merck Milipore, Tokyo, Japan). Then, 24 h after the change, the rats were subjected to food and water deprivation for 24 h and an SPT was performed. Two drinking bottles (200 mL of 1% w/v sucrose solution and 200 mL of pure water) were placed in the cage and the rats were allowed to freely drink for 24 h. The consumption (g) of sucrose solution and water for 24 h was measured and the sucrose preference was calculated using formula (1).

Sucrose preference (%) = \frac{sucrose consumption (g)}{sucrose consumption (g) + water consumption (g)} \times 100

(1)

Open Field Test (OFT)

We performed an OFT in a quiet (<60 dB) room between 11:00 and 13:00 on days 2, 9, 16, and 23 according to a previous study. We used a plastic apparatus (40 cm high) in which the pearl grey floor (100×100 cm) was divided into 25 squares (each 20×20 cm) by grey lines. Each rat was gently placed in the center of the apparatus and the behavior was video-recorded for 6 min. After the OFT on each rat was completed, the apparatus was cleaned with water and 70% vol% ethanol, and subsequently dried.

The occurrences of grid line crossings, rearing, and defecation in the recorded video (5 min; minutes 1 to 6) were counted to assess the depression-like symptoms.

$^{1}H$-NMR Spectroscopy of Tissue Samples

NMR Sample Preparation

Following the completion of the fourth OFT, rats were guillotined under diethyl ether anesthesia. The brain was promptly removed and the hippocampus was extracted by dividing the brain along the longitudinal fissure and rapidly frozen in liquid nitrogen. The frozen hippocampal samples were stored at −45°C until measurement by NMR spectroscopy. To ensure that the postmortem changes in metabolites remained uniform with minimal differences between the rats, this process was completed within a defined time period (10–15 min). In particular, the site analyzed in the current study was the hippocampus, because it represents an important component of the limbic system that governs emotions, and has also been the focus of clinical studies on depression.

NMR Apparatus and Sample Preparation

We used a Varian INOVA-700 NMR instrument at 699.7 MHz as the $^1$H frequency, equipped with a FASTNANO™ probehead (Agilent Technologies, Santa Clara, CA, U.S.A.). To allow detection, 43 µL deuterated water containing 2.5 mM sodium 3-(trimethylsilyl)-1-propanetriyl,1,1,2,2,3,3-d$_6$-sulfonate (DSS-d$_6$) was added to each hippocampal tissue sample (wet weight: 10–20 mg). The sample was manually homogenized with 20 rotations of a pestle (polypropylene) in a microtube. After the whole volume of the brain homogenate was transferred into a 43-µL glass cell with a Pasteur pipette, the cell was set into the 4-mm-outer-diameter zirconium oxide rotor. This process was repeated for each hippocampal sample. The rotors were then loaded into the NMR spectrometer.

Measurement Conditions

The parameters of the NMR spectrometer were set as follows: 90° pulse width, 7.80–8.00 µs (set for each sample by measurement); relaxation delay: 2.000 s; number of data points: 32 k complex; observation width: 8389.3 Hz; number of scans: 128; and rotation speed: 5000 Hz. The pre-saturation sequence was used to eliminate the water signal. We paid close attention to keeping the changes in metabolites nearly uniform between samples; accordingly, the measuring temperature was maintained at 298 K to narrow the changes in metabolites in the brain tissue caused by the change in temperature during measurement.

This operation was performed using VnmrJ software (Ver. 4.0, Agilent Technologies). With an aim for metabolite assignment, the Carr-Purcell-Meiboom-Gill (CPMG) sequence was used to acquire spectra under the same conditions.

Data Analysis of NMR Spectra

Assignment of Metabolite Signals

After Fourier transformation of all the acquired free induction decays, we manually performed phase correction and baseline correction using the VnmrJ software. The assignment of NMR data to metabolites was performed by comparison with the Chenomx NMR Suite (Ver. 8.1, Chenomx Inc., Edmonton, AB, Canada) and previously reported spectra.

Multivariate Analysis

For multivariate analysis of NMR-derived data, all the acquired free induction decays were zero-filled to 32 k using Alice2 for Metabolome software (Ver. 2; JEOL, Tokyo, Japan) and the absolute values were differentiated following Fourier transformation. The chemical shift range of 0.20–10.00 ppm (excluding the range of the water signal: 4.70–4.90 ppm) in the acquired NMR spectra was integrated in 0.04 ppm buckets to obtain 239 variables. Each bucket was then normalized to give a total integrated area of 100. The obtained integrated values were used to test the parameters in the multivariate analysis. A multi-step PCA was applied to remove the components that changed rapidly and markedly in order to explore the characteristics of the metabolites in depressive disorder.

Changes in Major Metabolites

We used a Student’s $t$-test to analyze the difference in the mean values of each bucket between the control and CS groups. $p<0.05$ was considered to be statistically significant. Theses analyses were performed using R version 2.15.2 software (R Foundation for Statistical Computing, Vienna, Austria) for Windows®.

RESULTS AND DISCUSSION

Evaluation of Depression Based on Body Weight, SPT, and OFT

The changes in body weight in rats are shown in Fig. 2a. The body weight in the control group was significantly higher on day 7 compared to that in the CS group ($p<0.01$). Body weight increased on days 14 and 21 over time in the control group, whereas no increase was observed in the CS group ($p<0.01$).

SPT is used to assess depressive states in animals as an index of “anhedonia.” Owing to the imposition of stress over time, the sucrose preference decreased in the CS group and was significantly lower on day 22 compared to the control group ($p<0.05$) (Fig. 2b).

The OFT is considered to reflect “locomotor activity,” “exploratory behavior,” and “fear/anxiety” in depressive states in laboratory animals. These depressive states can be assessed...
by counting “crossing numbers” as “locomotor activity,” 27) “rearing numbers” as “exploratory behavior,” and “defecation numbers” as “fear/anxiety.” The crossing, rearing, and defecation numbers during the period of exposure to the chronic stress are shown in Figs. 2c, d, and e, respectively. Both the crossing and rearing numbers were significantly decreased by the imposition of chronic stress on day 9 in comparison to the control group, whereas the defecation numbers were increased. As the significant differences in all the measurements of behavioral tests between the control and CS groups were observed following the imposition of stress for 23 d, we judged that a model of depression-like symptoms had been successfully constructed.

**HRMAS-1H-NMR Spectra of Hippocampal Tissue** The assignment of signals using the Chenomx NMR Suite and previously reported spectra as refs. 22 and 23) led to the assignment of 12 metabolites (Fig. 3). Of these, four (alanine, glutamate, glutamine, and aspartate) were significantly de-
creased by stress compared to the control group and three (N-acetylaspartate (NAA), myo-inositol, and creatine) were significantly increased (Table 1). Notably, alanine, glutamine, and glutamate have been previously reported to represent potential biomarkers of depressive disorder.\(^{28}\) Alanine was shown to decrease because of chronic stress\(^{29}\) and glutamate and glutamine were shown to be involved in ketogenesis and glucogenesis in response to xenobiotic stimuli.\(^{30}\) Alanine forms oxaloacetate from pyruvate, aspartate forms oxaloacetate, and glutamine and glutamate form \(\alpha\)-ketoglutarate, which are used for energy production in the tricarboxylic acid (TCA) cycle. The significant decrease in the CS group of amino acids involved in energy metabolism may suggest the existence of changes in energy metabolism owing to the abnormal TCA cycle caused by chronic stress. In addition, Ni \textit{et al.} have reported that the changes in alanine, NAA, aspartate, and glutamate levels in the hippocampus and the striatum of a rat model of depression differed depending on the site.\(^{21}\) Therefore, in the case of metabolites that showed significant changes in the current study, potential alterations in other regions of the brain may have differed from those in the hippocampus. Consistent with this finding, Wang \textit{et al.} and Tian \textit{et al.} reported that body weight, which is closely related to energy metabolism, increased more gradually in rats exposed to chronic stress compared to non-exposed rats, and that this phenomenon was caused by decreased food intake.\(^{15,31}\) Furthermore, Allard \textit{et al.} showed that in rats exhibiting enhancement of the hypothalamic–pituitary–adrenal axis, a characteristic depression-like symptom, the density of neurons in the brain producing orexin, which is a protein controlling feeding behavior, decreased.\(^{32}\) In addition, Ito \textit{et al.} demonstrated that the orexin levels in the brain were decreased by exposure to stress.\(^{33}\) Similarly, the orexin levels in the brain of patients with depressive disorder who had committed suicide were reported in a clinical study to be significantly lower compared to those in patients with adjustment disorder or dysthymia.\(^{34}\)

Specifically, the orexin neurons in the brain control the homeostasis of energy balance and nonessential amino acids have been shown to be involved to a greater degree in the

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Table 1. HRMAS-\(^1\)H-NMR Data and Assignments of the Metabolites in Rat Hippocampal Tissue

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Chemical shift (ppm)(^a)</th>
<th>Changed(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>1.32, 4.10</td>
<td>—</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.47, 3.77</td>
<td>↑*</td>
</tr>
<tr>
<td>GABA</td>
<td>1.90, 2.29, 3.01</td>
<td>—</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.91</td>
<td>—</td>
</tr>
<tr>
<td>NAA</td>
<td>2.01, 2.49, 2.68, 4.40</td>
<td>↑**</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2.04, 2.12, 2.33, 3.76</td>
<td>↓*</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.14, 2.43, 3.77</td>
<td>↓*</td>
</tr>
<tr>
<td>Aspartate</td>
<td>2.68, 2.80, 3.89</td>
<td>↓*</td>
</tr>
<tr>
<td>Choline</td>
<td>3.19, 3.51, 4.05</td>
<td>—</td>
</tr>
<tr>
<td>PC</td>
<td>3.21, 3.58, 4.16</td>
<td>—</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>3.27, 3.52, 3.61, 4.05</td>
<td>↑*</td>
</tr>
<tr>
<td>Creatine</td>
<td>3.03, 3.92</td>
<td>↑*</td>
</tr>
</tbody>
</table>

\(^a\) Bold letters indicate that these peaks were visually assigned in Fig. 3. \(^b\) ↑ or ↓ represent an increase or decrease of the metabolite as compared with the control group. \(^*\) and \(^**\) represent \(p<0.05\) or \(p<0.01\) as compared with the control group.

GABA, \(\gamma\)-aminobutyrate; PC, phosphocholine.
activation of orexin neurons than essential amino acids.\(^{35}\) Alanine, glutamate, and aspartate, which were significantly decreased in the CS group in our study, comprise nonessential amino acids, suggesting that the orexin levels in the brain were decreased by exposure to stress, thus causing a decrease in food intake and a concomitant decrease in body weight. In addition, NAA, which is produced in the mitochondria and serves as a marker of neuronal density, was reported to be significantly increased in the hypothalamus in chronically stressed rats,\(^{31}\) which parallels the results from hippocampal tissue observed in the current study. Notably, it has been shown that elevated NAA causes a compensatory increase in amino acids owing to the transport of amino nitrogen from the mitochondria to the cytoplasm.\(^{36}\) In turn, creatine, another metabolite elevated in CS rats, plays an essential role as an energy source in the brain. Consistent with this role, energy metabolism in the brain and changes thereof have been shown to be closely associated with the pathophysiology of depressive disorder in patients.\(^{37}\)

In the event of an external stressful stimulus, allostasis occurs through the initiation of physiological responses and coping behaviors to achieve adaptation of the living organism to the environment, whereas allostatic overload leads to chronic disease.\(^{38}\) In our rat model of depression-like symptoms, the energy-producing activity in the TCA cycle was abnormal, which is considered to have caused the increases in NAA and creatine in order to complement the loss of energy resulting from disease development. In turn, this likely resulted in a compensatory enhancement in the activity of alternative pathways that produce energy required for maintaining viability.

In addition, because of the possibility that food deprivation or sucrose intake represent factors that may influence the metabolite profile in the hippocampus or that such changes may impact behavior, we determined the correlation coefficients in the control group between sucrose intake and the integrated values for metabolites that showed significant changes. These assessments revealed no statistically significant correlation for any of the 7 metabolites (data not shown), which indicates that sucrose intake had little effect on alterations in hippocampal metabolites.

**Characterization of Hippocampal Samples by Non-targeted Multistep PCA**  In the PCA of 239 variables, the first (PC1) and second (PC2) principal components contributed 43.8 and 26.6%, respectively (cumulative rate: 70.4%). No clear separation between the CS and control groups was observed in the PCA score plot. Therefore, we applied a multi-step PCA, which allows the discovery of changes in metabolites that were hidden owing to extreme signals by excluding factors whose loading values are large when represented by variables in a loading plot.\(^{8,24}\) As we had previously experienced that the lactate and acetate signals exhibited large changes within the NMR-MP using urine and serum, we first excluded signals at 1.30 and 1.34 ppm (lactate) as well as at 1.90 ppm (acetate) to obtain 236 variables. Next, in order to avoid the contribution of strong signals derived mainly from sugars and aldehydes, the signals in the region of 4.00–10.00 ppm were removed to eventually obtain a two-dimensional score plot and loading plot of 92 variables (Fig. 4).

Figure 4a shows the score plot and Fig. 4b shows the loading plot. PC1 and PC2 contributed 69.5% cumulatively, which was sufficiently high to explain the characteristics of the metabolite changes in the samples. We could observe the two clear classes of the control and CS groups in Fig. 4a. NAA (2.02 ppm) was shown to represent the variable that most defined the distribution in the score plot in Fig. 4b.

In order to verify whether the two-dimensional coordinate in the score plot reflected the disease stages of the depression-like symptoms, the correlation coefficients between the behavioral scores and the PC1 as well as the PC2 score were computed using R version 2.15.2 (Table 2). We identified high negative correlations between the PC1 score in the score plot and body weight ($r = -0.805$), SPT ($r = -0.738$), and crossings ($r = -0.658$). The PC1 score was also negatively correlated with rearing ($r = -0.418$) and positively with defecation...
This suggests that the utilization of NMR-MP may enable the assessment of depressive disorder as intrinsic changes in metabolites in the brain. The present study allowed an exploration of the degree of depression-like symptoms. Thus, the score plot obtained in the pharmacological tests and reflected the degree of combined effects of stress. Therefore, our results demonstrated that the score plot is representative not of the assessment of the intensity of the stress. Therefore, our results demonstrated that the score plot is representative not of the assessment of the intensity of the stress. Therefore, our results demonstrated that the score plot is representative not of the assessment of the intensity of the stress.

In addition, the behavioral pharmacological scores of CS09, exhibiting the highest PC1 value; Con05, exhibiting the lowest PC1 value; and CS03, Con02, and Con08, which were placed in the border between the CS and control groups, were compared to assess the degree of each depression-like symptom (Table 3). The PC1 values were Con05 < Con08 < CS03 = Con02 < CS09, whereas the body weights were CS09 < CS03 < Con02 < Con08 = Con05, exhibiting a reversed order. The SPT values of the rats placed in the border were CS09 < Con02 < CS03 < Con05 = Con08, and thus were ordered differently than the body weight. The crossing values were CS09 < Con05 < CS03 = Con02 < Con08. Con08 and Con02, which were placed near the border with the CS group in the score plot, were ranked close to CS03 in the CS group in all three items of depression-like symptoms. Therefore, the types and degrees of depression-like symptoms caused by exposure to stress were not necessarily uniform, but diverse. It can readily be conjectured that even when rats are exposed to the same stress, the intensity of the phenotype (symptoms) varies in each rat owing to the expression of individual responses to the stress. Therefore, our results demonstrated that the score plot is representative not of the assessment of the intensity of only a single depression-like symptom, but rather of a comprehensive assessment.

The score plot representing changes in the brain metabolites showed correlations with several measurements of behavioral phenotypes and reflected the degree of combined depression-like symptoms. Thus, the score plot obtained in the present study allowed an exploration of the degree of depressive disorder as intrinsic changes in metabolites in the brain. This suggests that the utilization of NMR-MP may enable the comparison of depressive disorder intensity to better understand its degree and progression.

**CONCLUSION**

In order to visualize the pathological conditions of depressive disorder caused by chronic stress, we applied a HRMAS-1H-NMR-based MP method for the assessment of changes in metabolites. Our study demonstrated that MP with multi-step sequence is a useful method for providing important information to better understand the degree of disease that can be applied to 1H-NMR spectra obtained from tissue samples. We clarified that in the hippocampus of the rat model of a depression-like state, 7 metabolites (alanine, NAA, glutamate, glutamine, aspartate, myo-inositol, and creatine) were significantly altered to better understand the degree of disease that can be applied to 1H-NMR spectra obtained from tissue samples. We clarified that in the hippocampus of the rat model of a depression-like state, 7 metabolites (alanine, NAA, glutamate, glutamine, aspartate, myo-inositol, and creatine) were significantly altered by imposing chronic stress. These data suggest an imbalance in energy metabolism in the brain.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**


**Table 2. Correlation between Principal Component and Behavioral Values**

<table>
<thead>
<tr>
<th>PC1</th>
<th>SPT</th>
<th>Crossings</th>
<th>Rearing</th>
<th>Defecation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BW)</td>
<td>-0.805</td>
<td>-0.738</td>
<td>-0.658</td>
<td>-0.418</td>
</tr>
<tr>
<td>PC2</td>
<td>0.169</td>
<td>-0.262</td>
<td>-0.256</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Pearson’s correlation coefficient is shown. 0.2 ≤ |r| < 0.4: low correlation, 0.4 ≤ |r| < 0.7: moderate correlation, 0.7 ≤ |r| ≤ 1.0: high correlation.

**Table 3. Behavioral Scores in Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>PC1 value</th>
<th>BW (g)</th>
<th>SPT (%)</th>
<th>Crossings (number)</th>
<th>Rearing (number)</th>
<th>Defecation (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS01</td>
<td>0.1579</td>
<td>256</td>
<td>56.74</td>
<td>14</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>CS03</td>
<td>0.1360</td>
<td>274</td>
<td>80.33</td>
<td>28</td>
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<td>4</td>
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<tr>
<td>CS06</td>
<td>1.0543</td>
<td>249</td>
<td>65.86</td>
<td>23</td>
<td>14</td>
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<tr>
<td>CS07</td>
<td>1.2644</td>
<td>269</td>
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<td>3</td>
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<td>CS09</td>
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<td>47.93</td>
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<td>5</td>
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<tr>
<td>CS10</td>
<td>0.6795</td>
<td>255</td>
<td>22.67</td>
<td>12</td>
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<td>4</td>
</tr>
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<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Con02</td>
<td>0.2188</td>
<td>314</td>
<td>63.07</td>
<td>31</td>
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<td>0</td>
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<tr>
<td>Con04</td>
<td>-1.6918</td>
<td>324</td>
<td>95.73</td>
<td>71</td>
<td>19</td>
<td>5</td>
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<tr>
<td>Con05</td>
<td>-1.7875</td>
<td>329</td>
<td>93.69</td>
<td>20</td>
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<tr>
<td>Con08</td>
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<td>97.71</td>
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<td>Con11</td>
<td>-0.8209</td>
<td>327</td>
<td>90.71</td>
<td>28</td>
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<tr>
<td>Con12</td>
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<td>96.83</td>
<td>54</td>
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</tbody>
</table>

(r=0.471). The PC2 score was weakly negatively correlated with SPT, crossings, and defecation. Therefore, the first axis was interpreted to represent a comprehensive indicator including depression-like symptoms.


