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# Metabolic profiling reveals disorder of amino acid metabolism in four brain regions from a rat model of chronic unpredictable mild stress

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**Abstract Chronic stress is closely linked to clinical depression, which could be assessed by a chronic unpredictable mild stress (CUMS) animal model. We present here a GC/MS-based metabolic profiling approach to investigate neurochemical changes in the cerebral cortex, hippocampus, thalamus, and remaining brain tissues. Multi-criteria assessment for multivariate statistics could identify differential metabolites between the CUMS-model rats versus the healthy controls. This study demonstrates that the significantly perturbed metabolites mainly involving amino acids play an indispensable role in regulating neural activity in the brain. Therefore, results obtained from such metabolic profiling strategy potentially provide a unique perspective on molecular mechanisms of chronic stress.**


*Keywords:* Chronic unpredictable mild stress; Metabolic profiling; GC/MS; Multi-criteria assessment; Multivariate statistics; Amino acid

## 1. Introduction

Depression is a serious public mental but treatable problem, affecting about 12% of women and 7% of men annually in USA [1]. It interferes often with normal feelings and behavior in people's daily life, and causes severe psychological pain for the patients and their families. Current treatments for clinical depression commonly involve psychotherapy and antidepressant medications. The most popular types of these antidepressant drugs are selective serotonin reuptake inhibitors (SSRIs), e.g., fluoxetine, citalopram, sertraline and several others [2]. Chronic unpredictable mild stress (CUMS), a well-validated animal model, has been used widely for studying clinical depression as well as evaluating antidepressant effects of diverse drugs [3,4]. Much of the work has been done successfully in individual gene expression, protein structure and function, as well as biochemical studies on sympathetic nervous system (SNS), hypothalamic–pituitary–adrenocortical (HPA)-axis, noradrenergic and immunological systems, etc. [5–10]. Recently, the emerging metabonomics or metabolomics [11,12] has gradually studied the intricate relationship between acute and/or chronic stress and certain crucial endogenous metabolites [13–17]. Such metabolic profiling technology has been increasingly used as a versatile tool for the discovery of molecular biomarkers in many areas such as monitoring the chemical-induced toxicity in organs, diagnosing or prognosing clinical diseases, exploring the potential mechanism of diverse diseases, and assessing therapeutic effects of drugs [18–21]. Relying on the global metabolite changes in a given biological species, metabol/nomics requires little or no prior knowledge on a certain disease. Thus, it potentially provides not only a means of verifying the fragmentary findings from a great deal of individual previous research, but also a promising opportunity to generate novel hypothesis for addressing the molecular mechanisms of diseases, ultimately towards a comprehensive understanding of physiopathological outcomes of an organism in response to xenobiotic stimuli and/ or genetic modification.

Biofuilds such as urine and plasma have been heavily used in metabol/nomic studies because they are minimally invasive to the animals or human and primarily reveal an overall metabolic state of the given organism [22]. By comparison, the tissue samples can offer a unique perspective on localized metabolic information. As the brain is a highly complex system encompassing a broad array of mutually interacting metabolites with varied chemical properties and specific biological functions, metabolic profiling of brain tissue samples will yield beneficial knowledge most related to neural activity of central nervous systems (CNS) [23–25]. Yet no such study has been fully initiated to monitor neurochemical changes in a CUMS model.

Recent metabolic profiling technology has successfully applied high-throughput analytical tools (e.g., NMR, nuclear magnetic resonance; MS, mass spectrometry) to analyze

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*Abbreviations:* NMR, nuclear magnetic resonance; MS, mass spectrometry; GC/MS, gas chromatography/mass spectrometry; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares project to latent structures-discriminant analysis; MCA, multi-criteria assessment; VIP, variable importance in the projection; CUMS, chronic unpredictable mild stress; SNS, sympathetic nervous system; CNS, central nervous systems; HPA, hypothalamic–pituitary–adrenocortical; ECF, ethyl chloroformate; $CIJF_{jk}$, jack-knifed confidence interval; NAA, *N*-acetyl aspartate; BCAAs, branched-chain amino acids

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various biological samples and utilized multivariate statistics (e.g., PCA, principal component analysis; PLS, partial least squares projection to latent structures) to extract meaningful biological information from the resultant complex and huge data sets [26,27]. Variable selection is an important step in multivariate analysis that can apparently enhance our understanding and interpretability of multivariate models and commonly referred to VIP statistics, loading weights, and correlation coefficients [28–30]. However, the practical use of these methods relies mostly on the experimental designs and purposes (e.g., animal or human studies, biomarker identification or pathway analysis), the size of samples, and preference of researchers as well [31,32]. A strict approach for selecting significant and reliable variables should likely be a combination of multiple criteria. For instance, the newly proposed S-plot combines both covariance and correlation deriving from multivariate modeling [31]. But the exact criterion for each method in variable selection is hitherto not addressed thoroughly.

The primary goal of this work is to characterize neurochemical abnormalities in four discrete brain regions including cerebral cortex, hippocampus, thalamus, and the remaining regions from a rat model of CUMS. We applied a gas chromatography/mass spectrometry (GC/MS) technique to profile the brain tissue samples, and multi-criteria assessment (MCA) for multivariate statistics to select reliable variables accountable for class discrimination of metabolic profiles. The differential metabolites were verified partially by qualitative and quantitative analyses simultaneously. This work will not only provide a constructive protocol in choosing the most reliable and significant metabolites associated with a certain pathophysiological state when using metabolic profiling technology, but also expand our understanding of molecular mechanisms for diverse diseases.

## 2. Materials and methods

The schematic flowchart of the metabolic profiling strategy used in this study is illustrated in the Fig. 1.

### 2.1. Animal handling, sampling and sucrose preference test

The study was approved by national legislations of China and local guidelines. A total of twelve eight-week-old male Sprague–Dawley (SD) rats ($n = 6$ *per* group) was employed in this study and the animal experiment is described in the Supporting Materials.

### 2.2. Sample preparation, GC/MS assay, and data acquisition and pretreatment

The section was conducted as our previously described procedures [33] and is provided in the Supporting Materials.

### 2.3. Multivariate and univariate statistics

Multivariate statistics, including unsupervised PCA and supervised orthogonal partial least squares project to latent structures-discriminant analysis (OPLS-DA), was performed by SIMCA-P 11.0 software (Umetrics, Umeå, Sweden) [34,35]. The data set was mean-centered and pareto-scaled in a columnwise manner for all the multivariate modeling [36]. Mean centering calculates the average spectrum of the data set and subtracts that average from each spectrum, aiming to focus on the fluctuating part of data instead of the original value. Pareto scaling weighs each variable by the square root of its standard deviation, which amplifies the contribution of lower concentration metabolites but not to such an extent where noise produces a large contribution. PCA technique was initially used to reduce the high dimensional data sets into a two- or three-dimensional scores map

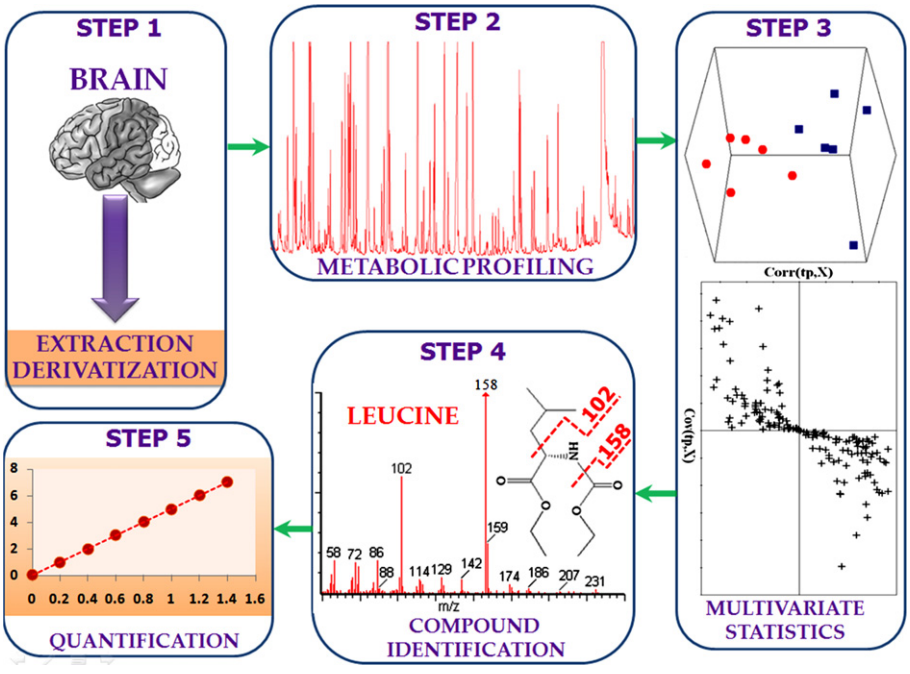


Fig. 1. Schematic flowchart of the metabolic profiling strategy used in this study. The homogenized tissue samples were extracted using ultrapure water and derivatized with ethyl chloroformate (ECF) (Step 1). The resultant derivatives were subsequently analyzed by a hyphenated technique – gas chromatography/mass spectrometry (GC/MS) (Step 2). Multivariate statistics was applied to extract meaningful information in the complex GC/MS spectral data (Step 3). Compounds with significant contribution to the variation of metabolic profiles between the CUMS-induced rats and healthy controls were identified in discrete brain regions using GC/MS spectral libraries including Wiley, NIST, NBS, etc., and further verified by reference compounds available (Step 4). Quantitative analysis of these verified metabolites was finally conducted by means of conventional calibration curves in order to accurately determine the concentrations of these metabolites in each brain region (Step 5).