Original Research

Broad and dynamic neurochemical alterations in the brain of alcoholic rats

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Ethanol is the active ingredient in alcoholic beverages. As ethanol consumption increases from zero to very high, it is still unknown which metabolites are present at different times and which are essential to normal functioning. In this article, we used an intermittent-access 20% ethanol drinking paradigm to make Wistar male rats voluntarily drink large amounts of ethanol for 10, 20, 30, and 50 days, respectively. A hydrogen-1 nuclear magnetic resonance approach was used to investigate the time-dependent neurochemical metabolites spectra in the hippocampus, striatum, nucleus accumbens and prefrontal cortex. Multivariate pattern recognition techniques were used to analyze the hydrogen-1 nuclear magnetic resonance spectra data. Metabolic profiling was obtained, differentiating the ethanol-treated and control rats. The ethanol-affected metabolites disrupted processes associated with neurotransmitters, oxidative stress, energy metabolism and amino acids. Together, our findings demonstrate broad, dynamic, and time-dependent endogenous metabolic alterations in rats treated with ethanol.

Keywords

Metabonomics; Metabolomics; Ethanol consumption; Hippocampus; Striatum; Nucleus accumbens; Prefrontal cortex; Rat

1. Introduction

Alcohol abuse studies on gene and protein expression and function are prevalent [1–3]. However, metabolites are more directly responsible for physiological and behavioral changes. Early studies using a metabolomics analysis of alcohol abuse revealed significantly disturbed metabolites [4] and different brain regions' metabolic variations [5]. However, alcohol addiction occurs over a long time. During the whole alcohol consumption process from day zero to addiction, which metabolites have an important function and how these metabolites change remains unknown.

Metabolomics can collect quantitative data on a wide range of metabolites at different points in time. This can provide critical information for understanding metabolic changes associated with disease progression. As a crucial research field, downstream of genomics and proteomics, metabolomics quantizes metabolites' global profile. It reflects the state of the organism's homeostasis and has been widely used in medical molecular diagnosis [6, 7]. Three powerful analytical techniques are available for metabonomic detection, including liquid/gas chromatography (LC/GC), mass spectrometry (MS) and nuclear magnetic resonance (NMR). Compared to MS, NMR, which has been used extensively since the 1970s, is a non-destructive and non-invasive technique and can identify a wide range of metabolites.

Alcohol abuse is characterized by a progressive increase in alcohol consumption, including intake, withdrawal, craving, and relapse. In previous studies, researchers adopted sucrose fading or water/food deprivation to initiate voluntary alcohol intake. However, ethanol consumption tends to decrease after removing the initiation factor [8]. Recent research suggests that repeated cycles of free-choice ethanol intake and withdrawal over several weeks lead to a gradual escalation of ethanol intake and preference, which reaches a stable baseline after several weeks [9, 10].

Earlier research using metabolomics to explore the brain metabolic profile of alcohol abuse in Wistar rats that voluntarily consume large amounts of ethanol showed that this significantly increased the rats' preference for ethanol [11]. In our research, ^{1}H NMR spectra was predicted to detect the global metabolic profiling in these brain regions: hippocampus, striatum, nucleus accumbens (NAc) and prefrontal cortex (PFC). Several main metabolic pathways are outlined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) mapper, including tricarboxylic acid cycle, amino acid metabolism and fatty acids metabolism.

2. Materials and methods

2.1 Ethanol intake procedures

With intermittent access to 20% alcohol and water, the rats voluntarily ingested large amounts of ethanol. The detailed ethanol drinking procedure was based on a previous study [11]. Briefly, the intermittent-access ethanol intake procedure consisted of many 2-day-sessions. At midnight, each Wistar rat was given a bottle of 20% (v/v) ethanol and

a bottle of tap water for the first day. On the second day, the bottle of ethanol was replaced with a bottle of water simultaneously. Rats were divided equally into two groups: an ethanol group given intermittent-access to 20% ethanol as described above, and a control group was given only access to tap water. Ethanol solution and tap water consumption were weighed daily, and the weight of each rat was also measured. Thus, ethanol and tap water consumption per kilogram of body weight were calculated every 24 hours.

2.2 Preparation of brain extracts

We prepared samples based on previous studies [12]. At the withdrawal period of a session, the different brain regions (hippocampus, NAc, striatum (mostly dorsal striatum excluded the NAc) and PFC) were suspended in ultrapure water containing methanol (add 4 mL per gram of tissue). The samples were homogenized by ultrasound. Chloroform was added at 2 mL per gram of tissue. Then chloroform and ultrapure water (2 mL per gram of tissue) were added into the suspension, mixed and homogenized again, and incubated for 15 min. Each sample was centrifuged at 1000 g for 10 min, and the top aqueous phase was collected. Use nitrogen to evaporate and dry. 580 uL D_2O was added to each tube of dry powder, and sodium (3-trimethylsilyl)-2, 2, 3, 3-tetradeuteriopropionate (TSP) with a final concentration of 0.01 mg/mL was added as the internal standard. Finally, the supernatant was transferred into 5 mm NMR tube for metabolite detection.

2.3 ^{1}H-NMR spectroscopy measurement

A Bruker Av-II spectrometer (Bruker Biospin, Germany) was used to collect spectral data at 300K. First, the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used to suppress the water signal, and a relaxation delay of 5 seconds was used to obtain the one-dimensional spectrum. In the process of data acquisition, the spectral width was defined as 12,335.5 Hz, the total pulse recycle delay was set as 7.66 seconds, and the acquisition time was 2.66 seconds. Subsequently, 64 free induced attenuation (FIDs) data points were collected as 64K. In a Fourier transform, the weighting coefficient of the FIDs data was set to the Gaussian line-broadening factor of 0.3-Hz; the maximum Gaussian position was 0.1. The baseline and phase correction of the spectral data were referenced to the TSP signal at 0.0 ppm.

2.4 Spectrum pattern recognition and analysis

A Fourier transform of the FIDs data, in MestReNova-6.1.1 software, was used to reduce the complexity of the NMR data and facilitate pattern recognition, to obtain concise information about the NMR spectrum. The data were then calibrated by phase and baseline. The data dimension reduction was set as the width of each region, 0.02 ppm, and the range was 0.5-9.5 ppm, dividing the spectrum into 419 segments. The region of 5.2-4.6 ppm represented the peak of water and was manually removed. The data were further normalized to eliminate differences between samples due to different tissue weights before the spectral data were sent into SIMCA-P (Version 11, Umetrics AB) for pattern recognition analysis. The ethanol samples were separated from the normal samples by analyzing NMR spectral data using principal component analysis (PCA). This was an unsupervised method of analysis. The supervised partial least squares discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA) methods were then used for further separation and filtering of the data. In the PLS-DA model, permutation analysis validation was set to 200 times. The reliability of the OPLS-DA model was assessed using R^2 (goodness of fit) and Q^2 (predictive power). R^2 ranged between 0 and 1, with 1 indicating a perfect-fit model. $Q^2 > 0.5$ represented good predictive ability, and > 0.9 excellent. In our study, the values of R^2 and Q^2 were high in the PLS-DA models of the ethanol and control groups. We further used the variable importance plots (VIP) values in the OPLS-DA model as the basis for selecting the peaks. Variables with a VIP value of > 1 were considered significant group discriminators. Also, each metabolite's chemical shifts were analyzed by unpaired Student's t-tests ($P \leq 0.05$). Only metabolites with VIP > 1 and $P \leq 0$ .05 were accepted as differences between groups. The chemical shifts were identified using the Human Metabolome Database (http://www.hmdb.ca/).

2.5 Statistical analysis

One-way ANOVA was used to analyze more than three sets of data. At the same time, the t-test was used to analyze two sets of independent data. $P \leq 0.05$ was considered significant.

3. Results

3.1 Ethanol intake in rats with intermittent-access alcohol drinking model

We first established an alcohol preference model in Wistar rats. The Wistar rats are a standard and reliable ratline for voluntary alcohol consumption models [13]. We used the intermittent-access drinking model to train rats' alcohol preference. The rats endured a total of 25 cycles (50 days) of 2-day-sessions. After calculating ethanol and tap water consumption per kilogram of body weight, the results showed there was a gradual rising in ethanol consumption (the folds relative to ethanol consumption on the first day, Fig. 1A) and a substantial decrease in water intake (the folds relative to water consumption on the first day, Fig. 1B). Next, Wistar rats' alcohol preference was evaluated with ethanol consumption ratio to water consumption on the ethanol days. The robust escalation in ethanol consumption was paralleled by a noticeable elevation in ethanol preference (Fig. 1C). Moreover, there was no difference in total liquid consumption per day between ethanol and water (Fig. 1D). Therefore, the animal model of ethanol abuse was markedly successful.

We induced voluntary increased alcohol intake using a model of intermittent access to 20% alcohol. However, it was clarified that only 50-80% of rats usually escalate to excessive alcohol drinking [10]. In the present experiment, alcohol consumption did not show a continuous increase. Some-

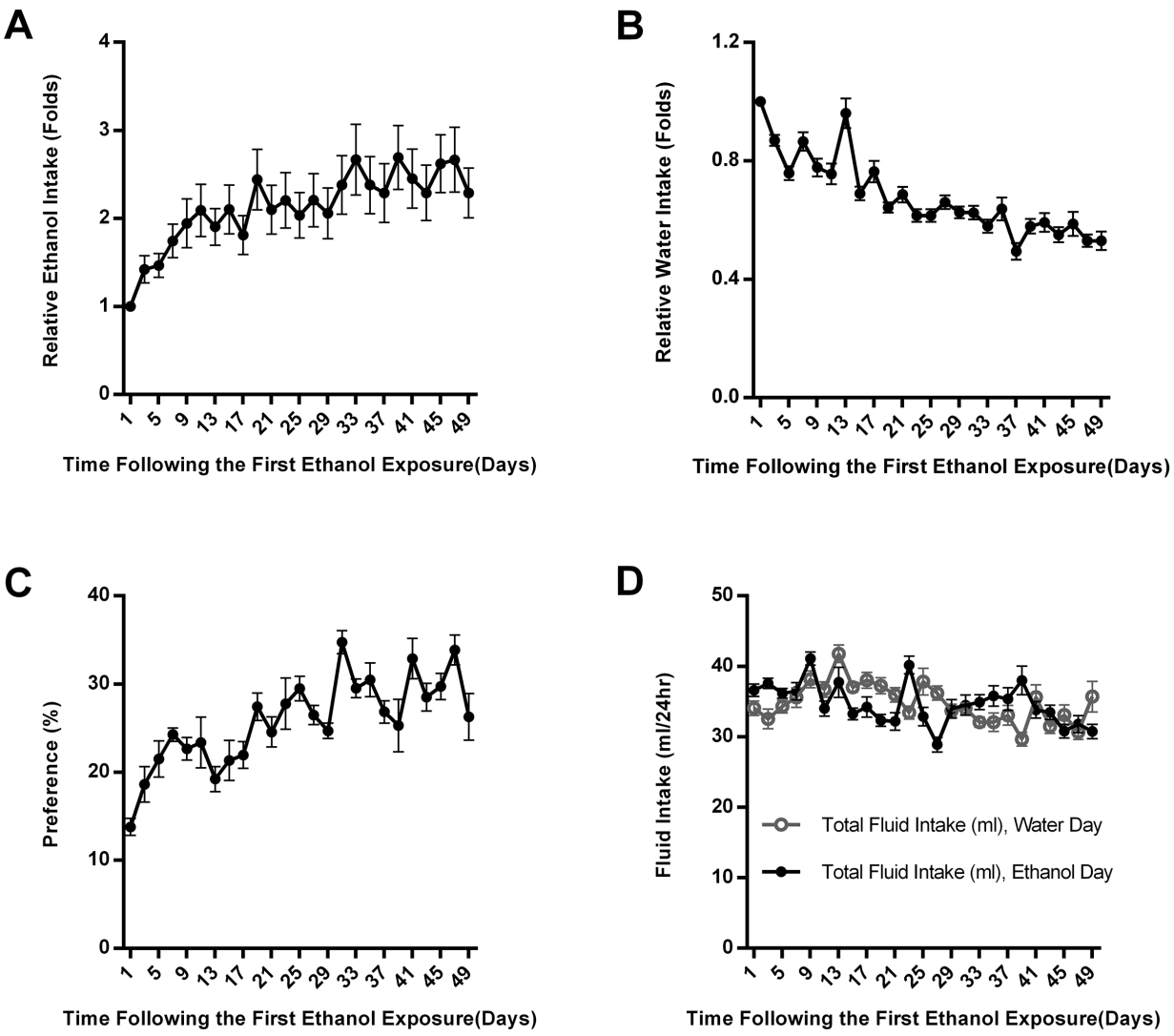

Fig. 1. Alcohol consumption in rats when given 20% ethanol intermittently. (A) After calculating ethanol or tap water consumption per kilogram of body weight, the results showed the alcohol drinking model induces a robust increase in relative ethanol intake (the folds relative to ethanol consumption on the first day); (B) A significant decrease in water drinking; (C) An elevated preference for ethanol (the ratio of ethanol consumption to water consumption on the ethanol days); (D) But there was no difference between the ethanol and water administration in total fluid consumption (mL/24 h). The data are presented as relative ethanol or water intake.

times, several animals showed low alcohol consumption, but another day might show a high consumption level during the next alcohol consumption. It was hard to distinguish these 2 subgroups while we collected the data from 0 to 50 days. For this reason, all animals (low and high drinkers) were included in the study.

3.2 ^{1}H NMR spectra analysis

The spectral data were reduced in dimensionality by MestReNova software. Peaks' locations were corrected using internal standard TSP. Then we manually removed the water peak and the internal TSP peak. Quantitative analysis was performed using total metabolite content. Finally, the distinctions of endogenous metabolite levels between ethanol and control groups' metabolites were identified using characteristic peaks in the database. Taking the hippocampus as an example, ^{1}H NMR spectra of ethanol and the control group are shown in Fig. S1.

PCA, as an unsupervised pattern recognition method, was first applied to the spectral analysis. Subsequently, a supervised PLS analysis method was used to further optimize the separation results. However, in the above two analysis modes, the first two principal components (PCs) were not significantly separated. As a result, orthogonal preprocessing was added to enhance the separation, after which each cerebral region of the two groups showed a significant difference in the PLS score plots. Notably, the OPLS model showed that there were significant differences in the PLS scores of each brain region in the 50-day ethanol intake group (Fig. 2A-D, left panels).

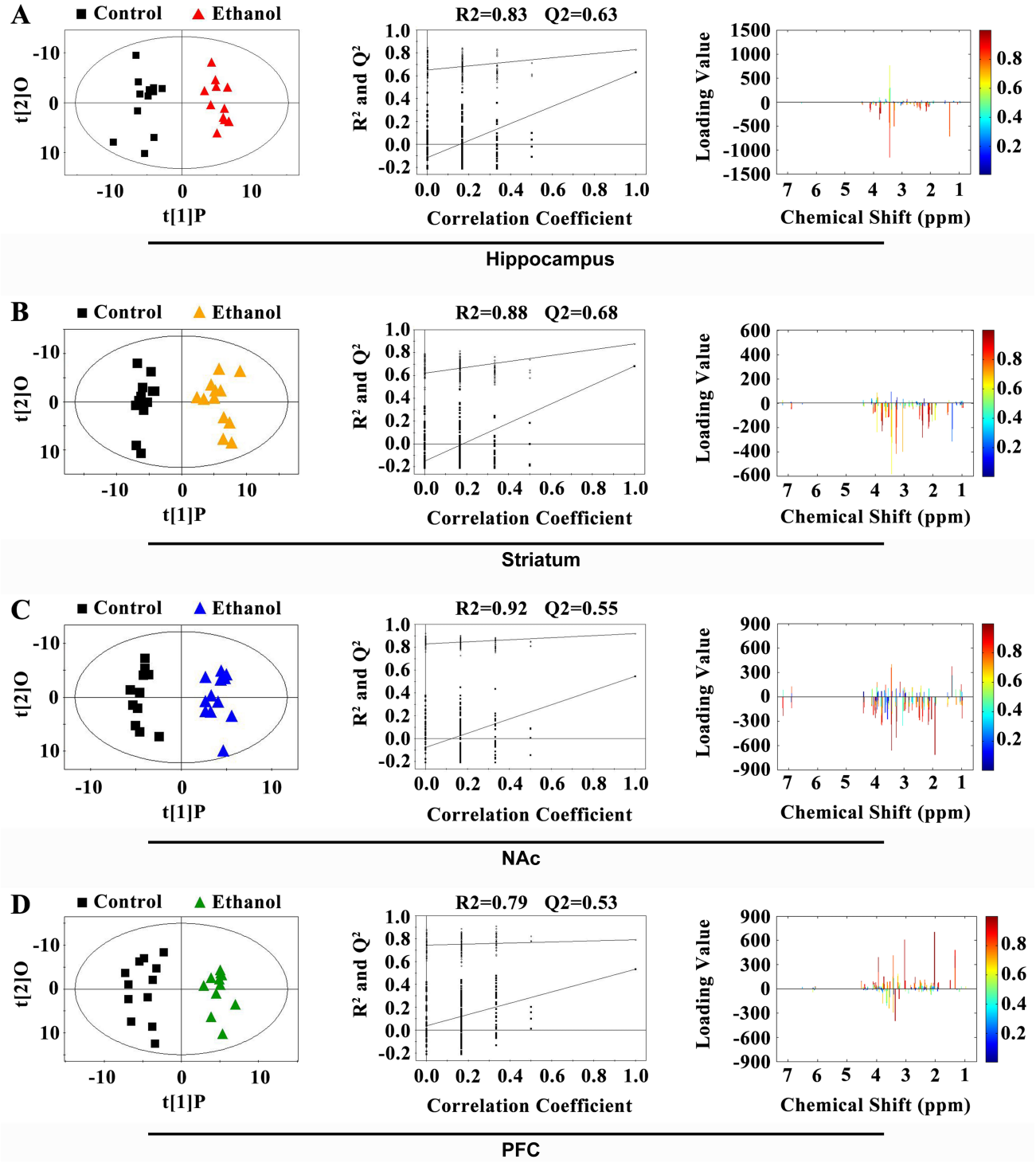

Fig. 2. Metabolite profiles of the four brain regions between 50-days ethanol-intake ($n = 12$) and the control rats ($n = 12$). (A, left) Orthogonal Partial Least Squares Discrimination Analysis (OPLS-DA) score plots of the hippocampus showed the separation of ethanol samples' metabolites from the normal samples. (A, middle) The quality and effectiveness of the OPLS-DA model were confirmed by a permutation method to obtain the R^2 and Q^2 values. Values close to 1 indicate models with high goodness-of-fit and predictive abilities. (A, right) The loadings were analyzed by clustering and colored accordingly by the MATLAB software. Compared with the normal control group, the increase of metabolites in ethanol-treated rats' hippocampus was represented by a positive peak. In contrast, the negative peak represented the decrease of metabolites in the ethanol-treated rats. (B-D) The corresponding spectral data analyses for the striatum, NAc and PFC regions.

Permutation analysis was used to estimate the validity of the PLS-DA model, using 200 permutations. The reliability of the OPLS-DA model was assessed using R^2 and Q^2 parameters. As shown in Fig. 2A-D (middle panels), model parameters of each brain region for 50-day ethanol-treated groups were as follows: NAc: $R^2 = 0.92$, $Q^2 = 0.55$; PFC: $R^2 = 0.79$, $Q^2 = 0.53$; striatum: $R^2 = 0.88$, $Q^2 = 0.68$ and hippocampus: $R^2 = 0.83$, $Q^2 = 0.63$. The R^2 and Q^2 values close to 1 indicated the models had high goodness-of-fit and predictive abilities.

We used NMR data from tissue samples to identify the significant differences in metabolites between ethanol drinking and control groups. We obtained the metabolite loadings plots and correlation coefficients through OPLS-DA analysis. According to the UV model's variable weight, the loadings were analyzed by clustering and colored accordingly. These results showed significant differences in metabolite categories. The positive signal indicated that the metabolites in the ethanol group's brain tissues were up-regulated compared with the control group. Negative signals indicate metabolite downregulation (Fig. 2A-D, right panels).

3.3 Dynamic metabolic profile of brain regions in ethanol-intake rats

Next, the significantly differentially abundant metabolites, which had VIP > 1 and $P < 0.05$ from *t*-tests, were summarized. Metabonomics was used to describe the metabolic profiling in the hippocampus, striatum, NAc, and PFC regions of rat brains modified by alcohol. Interestingly, we found different directions of metabolite changes. A few of the changed metabolites remained stable, as these were stable metabolic markers of ethanol exposure. However, most metabolites showed time-dependent changes in different directions. Some metabolites showed a time-dependent enhancement change: these metabolites were even more increased after prolonged ethanol exposure. Conversely, other metabolites showed a time-dependent reversal change: a decrease observed during prolonged ethanol treatment. An additional route observed included many newly differentially changed metabolites that appeared only after prolonged ethanol exposure. Subsequently, the representative differential metabolites of the hippocampus, striatum, NAc, and PFC were represented using box-and-whisker plots (Fig. 3).

We further analyzed the function of differential metabolites. We used the metabolic pathway mapper from the KEGG database to closely map changes in central metabolic pathways related to alcohol intake development (Fig. 4).

3.3.1 The modifications of neurotransmitters

Notably, in the hippocampus, ethanol intake leads to a significant decrease in neurotransmitters such as gamma-Aminobutyric acid (GABA), glutamate, and citrate. Glutamate and glutamine were also significantly reduced in the hippocampus, increasing ethanol consumption (Table S1). What is more, GABA and glutamine in NAc (Table S2), GABA in the striatum (Table S3), and glutamate and glutamine in PFC (Table S4) were also reduced at different stages of ethanol administration.

3.3.2 The changes in energy metabolism

Glucose, which is the primary source of energy metabolism, was decreased in NAc during ethanol administration and in the striatum after 50 days of ethanol administration. No change for glucose was discovered in the hippocampus and the PFC. Lactate, an alternative energy source in the brain, was decreased in the hippocampus, NAc and PFC regions. In the striatum region, lactate was increased after 20 days of ethanol administration.

Succinate, citrate, isocitric acid, and 2-oxoglutarate, which are involved in the citric acid cycle, were markedly modified by ethanol. Citrate, isocitric acid, 2-oxoglutarate in the hippocampus, isocitric acid and 2-oxoglutarate in the NAc, and citrate and isocitric acid PFC were reduced at different stages of ethanol administration. But citrate and succinate were increased in the striatum after 10-days of ethanol treatment.

3.3.3 Disruption of amino acids

We further examined amino acid metabolism and found many molecular changes, including valine, alanine, glycine, lysine, tyrosine, leucine, isoleucine, serine, threonine, aspartate, and arginine. Detected amino acids were all decreased in the hippocampus and NAc regions after ethanol administration. In the striatum, some amino acids, such as isoleucine, tyrosine, serine, valine, leucine and arginine, were markedly decreased by ethanol administration, but threonine and aspartate were increased. In the PFC, valine, aspartate, isoleucine, leucine and arginine were increased, whereas glycine, serine and threonine were reduced after ethanol consumption.

Also, we found mitochondrial and membrane-associated metabolites were disrupted. Phosphocholine, a kind of membrane ingredient, and N-Acetylaspartic acid (NAA), which is synthesized in mitochondria, were markedly elevated in the striatum NAc by ethanol administration, while these two metabolizes decreased in the hippocampus and the PFC. Myo-inositol was reduced significantly in the hippocampus, NAc, and PFC regions. However, in the striatum, it increased after ethanol consumption for 20 days.

3.3.4 Disturbances of antioxidants and other metabolites

Finally, the results showed glutathione, creatine, and taurine were markedly declined in the hippocampus, striatum, and NAc regions. These metabolites have prominent antioxidant properties. Other metabolites, such as 6-hydroxybutyric acid, acetate and dimethylamine, also significantly changed after ethanol treatment.

4. Discussion

The present study demonstrated that alcohol consumption dynamically changes the metabolites present in the hippocampus, nucleus accumbens, striatum, and prefrontal cor-

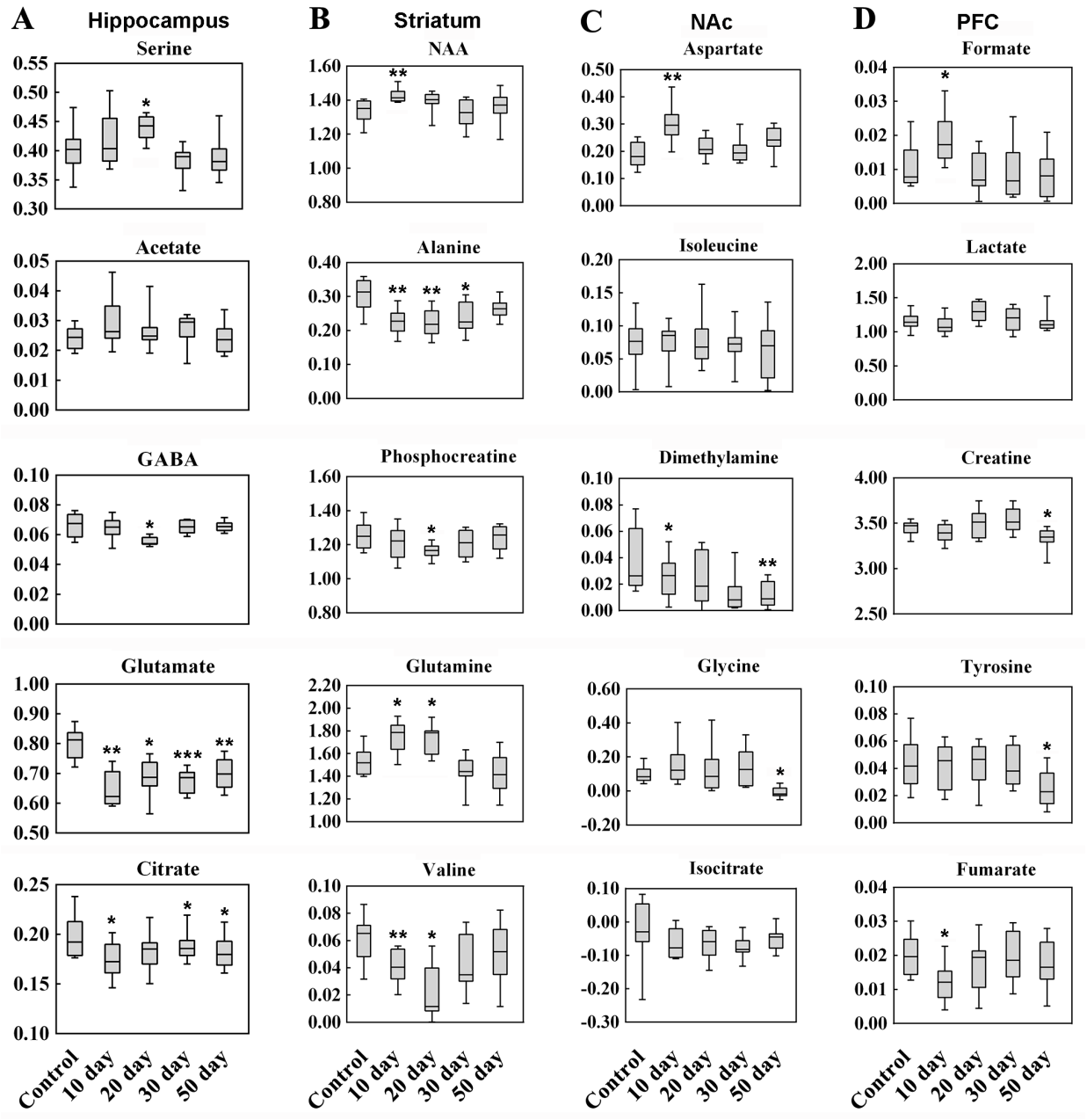

Fig. 3. The quantitative results of representative differential metabolites between ethanol-treated rats and control rats, shown as box-and-whisker plots. (A) Neurotransmitters and energy-metabolism-related metabolites were significantly altered in the hippocampus over the different stages of ethanol intake. (B) The primary metabolites that were modified in NAc were amino acid metabolites. (C) Amino acid and energy-metabolism-related metabolites were significantly altered in the PFC over the different stages. (D) The primary metabolites that were modified in the striatum were related to energy metabolism. The horizontal line in the middle of the box, bottom and top boundaries of boxes, lower and upper whiskers indicate 50^{th}, 25^{th}, 75^{th}, 5^{th} and 95^{th} percentiles, respectively. These data identified the time-dependent metabolic modification caused by ethanol. *, compared with the Control group.

tex regions of the brain. These changing metabolites are associated with neurotransmission, energy metabolism, oxidative stress, and amino acids.

4.1 Disturbance in neurotransmitters

Our results showed that alcohol intake affects the release of multiple neurotransmitters, including GABA, glutamine, and glutamate in various regions of the rat cerebrum. Among these neurotransmitters, glutamate is a major excitatory neurotransmitter, mostly found in astrocytes [14]. Glutamate levels can directly predict brain connectivity [15]. There are various glutamate receptors on cells, and they are known to have essential physiological functions. The ionotropic glutamate receptors (iGluRs) have voltage-gated cation channel activity, and the metabotropic glutamate receptors (mGlus) are coupled to GTP-binding proteins to mediate intracellu-

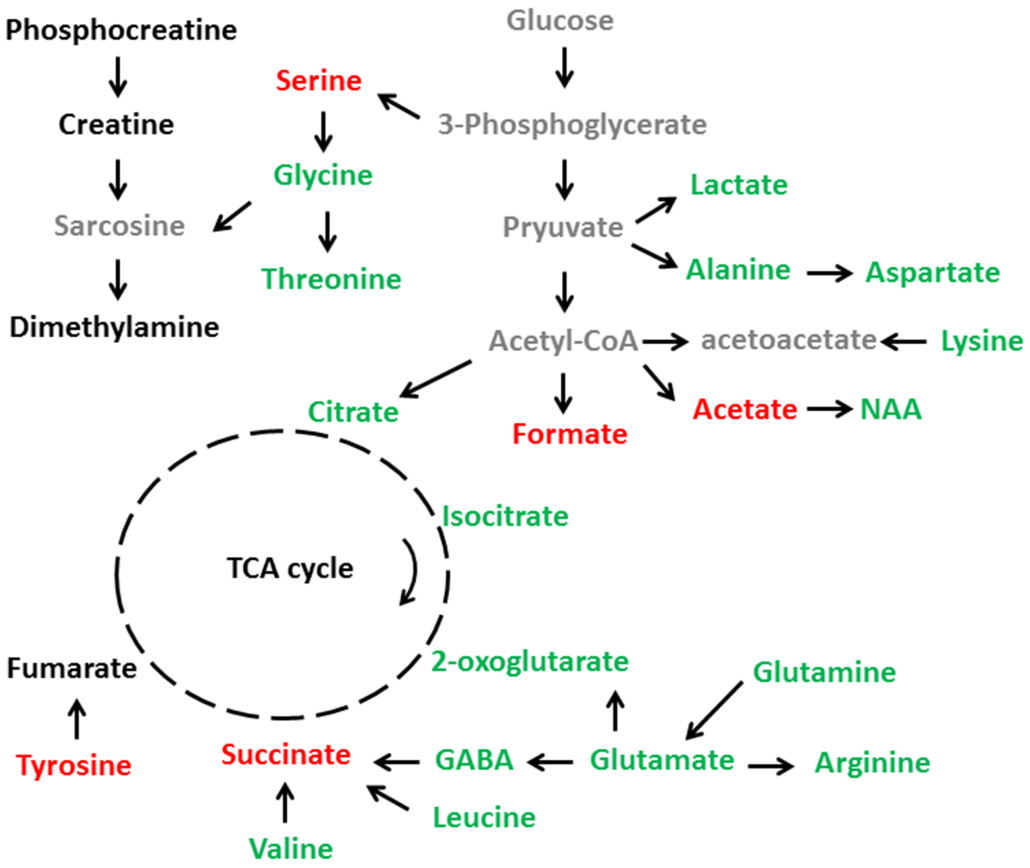

Fig. 4. The summary of ethanol-disturbed metabolic pathways. Grey font represents the metabolites that were ignored by the mass spectrometer. The green font represents the decreased metabolites in ethanol-treated rats. The red font represents the increased metabolites in ethanol-treated rats. The altered metabolites were characterized by amino acids, the tricarboxylic acid cycle (TCA), and neurotransmitters.

lar second messenger systems such as phospholipase C (PLC) and adenylate cyclase [16]. They have been reported in various pathological conditions, including Alzheimer's disease, amyotrophic lateral sclerosis (ALS), oxidative stress, and mitochondrial apoptosis [17].

Glutamate is also a precursor of GABA via the action of the enzyme glutamate decarboxylase and glutamine metabolism. GABA is the most important inhibitory neurotransmitter in the synapses of neurons. GABA and its receptor are widely concerned in depression and other diseases [18, 19]. In GABAergic neurons, glutamic acid is catalyzed by glutamic acid decarboxylase (GAD) to generate GABA [20]. Glutamine synthetase catalyzes glutamate to synthesize glutamine in astrocyte, which is then transported to the presynaptic neuron and is catalyzed by glutaminase to glutamate [21]. All these are neurotransmitter-related metabolites [22].

Decreased glutamate and GABA levels were previously reported in rats' whole brains after alcohol intake [4]. However, the details of these metabolite changes were unknown in addiction-related brain regions. In this study, the whole process of alcohol-abuse fingerprint results showed that GABA, glutamine, and glutamate decreased in the hippocampus region, glutamine and glutamate decreased in the PFC, GABA and glutamate decreased in the NAc, and GABA decreased in the striatum. Glutamate-related metabolites affect synaptic plasticity and affect the expression of addiction-related genes [23]. We suggest that these modified neurotransmitters may participate in alcohol addiction.

4.2 Oxidative damage

Our results suggest that alcohol consumption also alters the level of oxidative stress in the brain. NAA decreased in the hippocampus and the PFC of alcohol-consuming rats. Conversely, NAA increased in the NAc and the striatum. NAA is a marker of neurons' integrity and density, so NAA differences suggest that neurons differ across these brain regions. It has been reported that mitochondria are the leading synthesis site of NAA, which indicates that a change in NAA may be related to a decrease in adenosine triphosphate [24].

Moreover, other studies have found that the increase of dopamine and glutamate in the intercellular space can cause oxidative stress and neurotoxicity. This stimulation damages neurons, ultimately lowering dopamine and serotonin levels in the brain [25, 26]. In conclusion, NAA can be used as a reliable candidate molecule to evaluate alcohol-induced oxidative damage by reflecting nerve cells' viability. The different NAA changes across different brain regions indicate that alcohol-induced oxidative stress could affect different re-

sponses in these regions.

Decreased glutathione (in the hippocampus) was also observed following alcohol consumption. Glutathione is an essential small molecule antioxidant in the brain that defends against oxidative damage. Recent research has reported that glutathione is abundant in mitochondria and is involved in mitochondrial-related oxidative stress response in Alzheimer's and autism spectrum disorder [27, 28]. The diseases associated with oxidative stress often lead to a decrease in glutathione. Therefore, oxidative stress may play an essential role in the depletion of glutathione in the hippocampus. We also noticed a decrease in taurine in the hippocampus, the NAc and the striatum. Taurine is a neuroprotective amino acid with antioxidant properties and functions in apoptosis inhibition and calcium modulation [29]. Alcohol-induced dopamine release in the NAc can be inhibited by taurine [30]. Considering our results and previous research, decreased NAA, glutathione and taurine levels may suggest neuron injury and oxidative stress caused by alcohol. The underlying mechanisms and their functions need further confirmation.

4.3 Energy metabolism disturbance

Oxidative stress damages mitochondria and causes neurotoxicity. In the present study, oxidative damage likely decreased tricarboxylic acid (TCA) cycle products such as citrate, α-ketoglutarate, isocitric acid, and succinate hippocampus, PFC and NAc regions. This means the energy metabolism was blocked by alcohol abuse in these brain regions. However, lactate was raised in the striatum. The final metabolite of glucose for energy under cellular anaerobic conditions is lactate. Lactate is usually elevated in pathological changes in mammalian anaerobic conditions. Lactate levels increase instantaneously when neurons are activated because lactic acid provides energy to the neurons [31]. Our results suggest that an increase in lactate from alcohol intake leads to increased anaerobic metabolism. The increase in lactate in the striatum may indicate that alcohol may affect oxidative metabolism in the brain.

Recent studies have shown that the formation of addictive memories is related to synaptic transmission and morphology modifications. These modifications require a lot of energy: Creatine and other TCA cycle products are thought to play a crucial role in energy metabolism within the brain [32]. Accordingly, we demonstrated that the TCA cycle was found to decrease in the hippocampus, PFC and NAc regions of alcohol-consuming rats. This may indicate a severe energy deficit in the brain.

Abbreviations

^{1}H NMR, hydrogen-1 nuclear magnetic resonance; LC/GC, liquid/gas chromatography; MS, mass spectrometry; NAc, nucleus acumens; OPLS-DA, Orthogonal PLS-DA; PCA, Principal component analysis; PFC, Prefrontal cortex; PLS-DA, Partial least squares discriminant analysis.

Author contributions

LL and FFS designed the research study. LL, XLM and FFS performed the research. XLM and XH analyzed the data. LL and FFS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal protocols were approved by The Ethics Committee of Chongqing Medical University (No. 2017-0135).

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://jin.imrpress.com/EN/10.31083/j.jin.2021.01.254.

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