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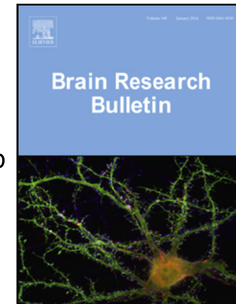

Untargeted metabolomics analysis of rat hippocampus subjected to sleep fragmentation

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Title Page

Title

Untargeted metabolomics analysis of rat hippocampus subjected to sleep fragmentation

Running title: Metabolomics of sleep-fragmented rat hippocampus

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Highlights

- ▪ Several metabolites and specific pathway are altered in the hippocampus by SF.
- ▪ The metabolite profiles vary according to the duration of SF.
- ▪ The alanine, aspartate, and glutamate metabolism pathway is the most altered pathway.

Abstract

Sleep fragmentation (SF) commonly occurs in several pathologic conditions and is especially associated with impairments of hippocampus-dependent neurocognitive functions. Although the effects of SF on hippocampus in terms of protein or gene levels were examined in several studies, the impact of SF at the metabolite level has not been investigated. Thus, in this study, the differentially expressed large-scale metabolite profiles of hippocampus in a rat model of SF were investigated using untargeted metabolomics approaches. Forty-eight rats were

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divided into the following 4 groups: 4-day SF group, 4-day exercise control (EC) group, 15-day SF group, and 15-day EC group (n=12, each). SF was accomplished by forced exercise using a walking wheel system with 30-s on/90-s off cycles, and EC condition was set at 10-min on/30-min off. The metabolite profiles of rat hippocampi in the SF and EC groups were analyzed using liquid chromatography/mass spectrometry. Multivariate analysis revealed distinctive metabolic profiles and marker signals between the SF and corresponding EC groups. Metabolic changes were significant only in the 15-day SF group. In the 15-day SF group, L-tryptophan, myristoylcarnitine, and palmitoylcarnitine were significantly increased, while adenosine monophosphate, hypoxanthine, L-glutamate, L-aspartate, L-methionine, and glycerophosphocholine were decreased compared to the EC group. The alanine, aspartate, and glutamate metabolism pathway was observed as the common key pathway in the 15-day SF groups. The results from this untargeted metabolomics study provide a perspective on metabolic impact of SF on the hippocampus.

Word count: 234

Keywords: sleep fragmentation, hippocampus, untargeted metabolomics, exercise control, metabolites

1. Introduction

Sleep is an essential process, and appropriate amounts of sleep are necessary to achieve normal body function. However, mounting evidence has shown that sleep continuity is also important for health, and decreased sleep continuity has been associated with several pathologic symptoms (e.g., increased inflammatory cytokines, elevated blood pressure, and impaired neurocognitive functions) and risk of chronic disease such as diabetes or cardiovascular disease (Dumaine and Ashley, 2015; Ekstedt et al., 2004; Qian et al., 2016;

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Ramesh et al., 2012). Sleep fragmentation (SF), defined as brief arousals that occur during sleep, can be induced by external factors such as bright light, high temperature or humidity, and noise during sleep. Moreover, it occurs frequently in patients with sleep apnea, chronic pain (Blagestad et al., 2012), periodic leg movements(Mancebo-Sosa et al., 2016), and asthma (Luyster et al., 2012). SF causes excessive daytime sleepiness (EDS), possibly by inhibiting cholinergic neuronal activity in the basal forebrain and elevating adenosine level (McKenna et al., 2007). EDS increases the risk of motor vehicle accidents (Ward et al., 2013) and decreases daytime functioning (Gooneratne et al., 2003; Stepanski, 2002). In addition, EDS is strongly associated with incident cardiovascular morbidity and mortality (Newman et al., 2000).

The hippocampus is a brain structure especially vulnerable to sleep disturbance in terms of morphological and functional aspects. In a recent experimental study, chronic sleep restriction (SR) resulted in a 10% reduction in hippocampal volume in rats, without an overall decrease in cortical thickness compared with controls (Novati et al., 2011). Human neuroimaging studies that examined the association between short sleep or disturbed sleep and hippocampal volume showed similar findings to those reported in animal studies (Joo et al., 2014; Taki et al., 2012). In animal studies, SF resulted in hippocampus-dependent learning and cognition deficits (Nair et al., 2011; Sportiche et al., 2010; Tartar et al., 2006). However, the appearance of negative impacts of SF varies depending on the duration of SF (Wallace et al., 2015). The possible mechanisms by which SF induces impairments include reduction in hippocampal neurogenesis (Guzman-Marin et al., 2007; Sportiche et al., 2010), increase of reactive oxygen species through upregulation and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and loss of N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation in hippocampal area CA1 (Tartar et al., 2010; Tartar et al., 2006).

Metabolomics is an emerging field of 'omics' research for global or system-wide metabolite profiles under a given set of conditions (Goodacre et al., 2004; Wishart, 2008), using analytical chemistry techniques such as nuclear magnetic resonance (NMR), chromatography, and mass spectrometry (MS)(Azad and Shulaev, 2018; Zhang et al., 2012). This field of research promises a better understanding of the pathophysiology of several chronic diseases and development of potential biomarkers (Ivanisevic and Thomas, 2018).

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Metabolomics has also been used for therapeutic monitoring and development of drugs (Puchades-Carrasco and Pineda-Lucena, 2017; Wei, 2011). Metabolomics is divided into two approaches (Patti et al., 2012), targeted and untargeted. Targeted metabolomics attempts to identify or quantitate defined individual metabolites (Roberts et al., 2012), whereas untargeted metabolomics investigates comprehensive profiles of all measurable metabolites using high-throughput methods (Vinayavekhin and Saghatelian, 2010).

Molecular changes caused by SF or sleep deprivation (SD) in genes and protein levels of rodent brains have been previously investigated. Reportedly, genes or proteins associated with cellular response to stress, energy metabolism, neuronal transmission, and synaptic plasticity were altered by SF or SD (Cirelli, 2006; Franco-Perez et al., 2012; Guzman-Marin et al., 2006). However, previous studies have several weaknesses regarding use of the appropriate animal model and limited methodologies. First, occurrence of long-term SD in humans is rare. Second, high-throughput gene expression analysis approaches cannot predict a post-translational modification of proteins, complicating direct interpretation of the function of expressed genes. Third, specific types of proteins are difficult to analyze using proteomic technologies (Chandramouli and Qian, 2009). However, alteration of metabolites reflect the actual activity of the cells; thus, the changes in the metabolome are more amplified than those in the transcriptome and the proteome (Urbanczyk-Wochniak et al., 2003). Moreover, the metabolome shows greater diversity and thus is closer to the phenotype of the biological system than the transcriptome and the proteome (Horgan et al., 2009). Therefore, in the present study, the changes of metabolites in the hippocampus, a brain region vulnerable to sleep disturbance, were investigated using untargeted metabolomics. This approach could provide a better understanding of the hippocampus including pathophysiology of hippocampus-dependent learning and cognitive impairments subjected to SF in terms of individual metabolites or specific metabolic pathways.

In this study, 4-day and 15-day SF models were used and the metabolite profiles were compared to the corresponding exercise control groups. We considered both the chronological criteria and the periods in which appropriate phenotypes can be expressed based on previous studies to determine the appropriate duration of the experiment. In terms of duration, acute SF generally refers to a one-night or several-night period in which sleep is interrupted, while chronic SF refers to a relatively long period of interrupted sleep (weeks or

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months). Indeed, electrophysiological data show that acute SF is different from chronic SF. Another study, with a different duration from our experimental period, have shown distinct effects on non-rapid eye movement (NREM) and rapid eye movement (REM) bout length during recovery periods in 3-day (acute) and 14-day (chronic) sleep-fragmented mice (Wallace et al., 2015). Other studies have also shown that 4-day and 15-day SF caused a reduction in hippocampal neurogenesis (Guzman-Marin et al., 2007) and deficits in hippocampus-dependent cognitive function (Nair et al., 2011), respectively. Thus, we chose 4-day and 15-day to distinguish the acute and chronic effects of SF on biochemical changes in hippocampus.

2. Materials and Methods

2.1 Animals

Seven-week-old male Wistar rats (Orient Bio, Korea) weighing 210–230 g were used in this study. Animals were maintained in a temperature-controlled room (24 ± 2°C) with alternating 12-h light and 12-h dark cycles (lights on at 8:00 a.m.) The rats had free access to food and water. After the 1-week acclimatization period, 48 rats were randomly divided into the following four groups: 4-day SF group (n=12), 4-day exercise control (EC) group (n=12), 15-day SF group (n=12), and 15-day EC group (n=12). All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Korea University College of Medicine (KUIACUC-2012-148).

2.2 Sleep recording and analysis

In separate experiments, electroencephalography (EEG) was recorded in the 4-day and 15-day SF and EC groups (n=4, each) to evaluate the suitability of the SF and EC models. Two pairs of EEG electrodes were implanted into the frontoparietal and lateral lobes and a pair of electromyography (EMG) electrodes (Pinnacle Technology Inc., Lawrence, KS, USA) was inserted into the neck muscles of rats. After surgery, the rats were recovered for 7 days. The rats were connected to a cable and acclimatized to the processes for 2 days before acquisition of baseline data. For 4-day group, we recorded and analyzed 24 hours of EEG and

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EMG at a sampling rate of 200 Hz on baseline, Day 1, Day 2, Day 3, and Day 4 of SF and EC procedures. For the 15-day group, 24 hours of EEG and EMG were recorded at baseline, Day 7, and on Day 15 of SF and EC procedures. Sleep data were semi-automatically analyzed using Sirenia software (Pinnacle Technology Inc., Lawrence, KS, USA), and were then confirmed by visual scoring to determine the percentage of sleep time by stage, bout number, and bout length. The duration of one epoch was set to 10 seconds. The frequency range for each waveform was defined as delta 0.5-4 Hz, theta 4-8 Hz, and alpha 8-13 Hz. A bout was defined as at least two consecutive 10-s epochs for a given state and ended with any single sate-change epoch.

2.3 Experimental procedures for SF and EC

Schematic experimental protocols are shown in Figure 1 (Fig. 1A and 1B). The forced exercise walking wheel system for rats was used to interrupt sleep in rats (Model number: 80805A, Lafayette Instruments, Lafayette, IN, USA), a previously validated method for the effectiveness of SF in mice (Yoon, 2012). Briefly, the rats were placed in an individual motor-driven wheel 7 days before the start of SF or EC procedure to acclimate to the novel environment. Experimental conditions for the 4-day and 15-day SF groups were as follows: activation time (wheel running): 30 s, stopped time (wheel not running): 90 s, wheel speed: 4.5 m/min, duration: 24 h/day. The condition consistently produced 30 awakenings per h, mimicking arousals observed in severe sleep apnea. To exclude the effects of non-specific movement, an additional EC group was used. The conditions for the EC group were 10 min on/30 min off, which offers the same amount of walking distance as for the rats in the SF group. The rats were weighted at baseline and on the day of sacrifice.

2.4 Sample preparation

On sacrifice day, the rats were deeply anesthetized using isoflurane and then decapitated using a rat guillotine. The hippocampi were excised, immediately snap-frozen in liquid nitrogen, and stored at -80℃ until further processing. The frozen hippocampi were cut into pieces approximately 0.7–0.8 cm x 1 mm in size using a surgical knife, transferred to a mortar chilled using liquid nitrogen, and homogenized. The pulverized samples were dissolved using 1.0 mL of cold methanol/acetonitrile/water buffer (5:3:2) and then transferred

to 1.5-mL microtubes. The centrifugation was performed at 28,756 g for 20 min at 4°C, and the supernatant was centrifuged under the same conditions for an additional 20 min to remove particulate matter. The supernatant was dried using a centrifugal concentration system (VS-802 Centra-vac, Vision Scientific, Bucheon, Korea), and the extract was dissolved in 50 μL of methanol/acetonitrile/water buffer (5:3:2). The entire sample preparation was finished in 3 h.

2.5 Liquid chromatography/MS (LC/MS)

The liquid chromatography/MS (LC/MS) analysis was performed as previously described (Jin et al., 2014). In brief, 5 μL of the dissolved sample was injected into the Agilent 1100 series high-performance LC (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) coupled with LTQ-XL high performance linear ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with an ESI source. The Xcalibur software (Thermo Fisher Scientific Inc.) was used to control chromatographic and mass spectral functions. In addition, for further MS/MS analysis, the samples were injected into the Agilent 1200 Infinity HPLC system coupled with electrospray ionization (ESI)-QTOF (Agilent Technologies). A Kinetex C18 column (2.6 μm, 100 × 4.6 mm; Phenomenex, Torrance, CA, USA) was maintained at 35°C for chromatographic sample separation. The mobile phases were 0.1% formic acid in deionized water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow rate of the mobile phase was set at 0.35 mL/min. Analytes were eluted with a mobile phase composed of 0.1% formic acid in water (A) and acetonitrile with 0.1% formic acid (B). The following gradient conditions were used: 0–14 min gradient 5–25% B, 14–19 min gradient 25–40% B, and 19–23 min gradient 40–95% B. For 6 min, 95% B solvent was maintained to return the gradient to the starting conditions, and column re-equilibration was performed for 6 min. Data were acquired in positive mode.

2.6 Data processing, multivariate analysis, marker identification, and pathway analysis

Before LC/MS data analysis, all metabolites in a sample were divided by the total observed number of ions in the sample to normalize the data. The LC/MS data were processed with MZmine 2.10 including data conversion and processing as previously described (Jin et al., 2014; Pluskal et al., 2010). Briefly, peaks were identified from LC/MS chromatograms using the local minimum search function, and a peak list was established by grouping ion intensities, matching *m/z*, and retention time. The peak list was exported to csv

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form and imported into MetaboAnalyst (Xia et al., 2015). Normalization and peak alignment were performed using the sum of all detected peaks, and the processed data were transferred into SIMCA-P version 11.0 programs (Umetrics, Umeå, Sweden) for multivariate statistical analysis. The principle component analysis (PCA) and the partial least squares discriminant analysis (PLS-DA) were performed to determine metabolic differences between the 4-day and 15-day SF and EC groups. The orthogonal projections to latent structure discriminant analysis (OPLS-DA) were performed to determine whole metabolic differences between EC and SF group regardless of experimental times (4-day or 15-day). All multivariate models were constructed in an iterative procedure until the predictability value (Q2) did not increase. The contributing metabolites for group discrimination were identified by comparing the *m/z* and MS/MS fragmentation patterns from the Human Metabolome Database (www.hmdb.ca), METLIN (http://metlin.scripps.edu), and MassBank database (http://www.massbank.jp). Metabolic pathway analysis was performed by MetPa analysis through MetaboAnalyst 4.0 (www.metaboanalyst.ca).

2.7 Statistical analysis

Differences in body weight and sleep parameters (percent of time spent in each stage, bout number, and bout length) between baseline and follow-up period in the same groups were determined by paired *t*-test. Differences in body weight and sleep parameters between EC and SF groups were examined by independent *t*-test for inter-group comparisons. All statistical analyses were conducted using SPSS version 20.0 (IBM, Chicago, IL, USA), and $P<0.05$ was considered statistically significant.

3. Results

3.1 Assessment of body weight and the effects of 4 days' and 15 days' SF or EC on sleep parameters

Body weight was measured at baseline and on Day 4 (4-day group) or Day 15 (15-day group) in the EC and SF groups. 4-day SF group had a slightly lower body weight than the 4-day EC group on the fourth day of experiment, but the difference was not statistically significant ($P = 0.054$) (Fig. 1C). There was no significant difference in body weight between

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15-day EC and 15-day SF groups at baseline (P= 0.240) and on Day 15 (P = 0.324) (Fig. 1D). Throughout the experimental period of the 4-day group, the percentages of time spent in Wake and NREM sleep did not differ significantly between baseline and follow-up or between the EC and SF groups (Fig. 1E and 1F). However, the time spent in the REM sleep stage was significantly reduced during the entire experiment in the SF group, compared to those in the baseline and corresponding EC groups (Fig. 1G). The average Wake and NREM bout numbers in the 4-day SF groups were significantly higher than those in the baseline and corresponding EC groups throughout the experimental period (Fig. 1H and 1I). However, the average NREM bout length decreased in the 4-day SF group on all days of fragmentation, compared to the baseline and corresponding EC group (Fig. 1J). In the 15-day group, the percentages of time spent in Wake, NREM, and REM sleep did not differ between baseline and follow-up or between the EC and SF groups (Fig. 1K-1M). However, the average Wake and NREM bout numbers in the 15-day SF group were significantly higher than those in the baseline and corresponding EC group while NREM bout length was lower on Day 7 and Day 15 (Fig. 1N-1P). A large number of Wake and NREM bouts but short NREM lengths indicate highly fragmented sleep. The EC group had no significant difference in the time spent in each sleep stage, the number of Wake and NREM bouts, and the NREM bout length compared to the baseline at all experimental periods, which shows well-preserved sleep continuity.

3.2 Metabolic differences between SF and EC groups

We performed multivariate statistical analysis and established a discrimination model (Fig. 2). The PCA and PLS-DA analysis showed group separation between SF and EC group, as well as clear discrimination on 4-day set and 15-day set (Fig. 2A and 2B). The PLS-DA analysis on SF and EC group from 4-day and 15-day showed much clear discrimination between 4-day SF and 4-day EC groups and 15-day SF and 15-day EC groups, with a Q2 value of 0.782 (R2X = 0.594, R2Y = 0.988) and 0.797 (R2X = 0.621, R2Y = 0.905), respectively (Fig. 2C and 2D). The significantly changed metabolites were selected with the statistical significance based on p-value and false discovery rate (FDR) adjusted p-value. All the considered data sets were visualized for a substantial separation of the two groups of samples; for instance, 4-day SF vs. 4-day EC and 15-day SF vs. 15-day EC (Fig. 3). Based on metabolite selection criteria, 66 and 444 markers were selected from the 4-day and 15-day data sets, respectively.

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3.3 Identification of metabolites significantly altered in SF groups

Among the selected markers, total 10 key markers that contributed to group separation between the overall SF and EC groups were identified including adenosine monophosphate (AMP), glycerophosphocholine (GPCh), hypoxanthine, jasmonic acid, L-aspartate, L-glutamate, L-methionine, L-tryptophan, myristoylcarnitine, and palmitoylcarnitine (PLC). Marker identification was performed by comparing the measured *m/z* value and the MS/MS fragmentation spectra of the main markers with the theoretical values of metabolites provided in the database (Supplementary Fig. 1 and Supplementary Table 1). The identified metabolites are summarized in Table 1 and represented in an S-plot (Fig. 4). According to the statistical significance based on p-value and FDR adjusted p-value, the metabolic changes in the 4-day group were neither so dramatic nor significant. Significant metabolic changes were observed only in the 15-day group. In the 15-day group, 9 markers were identified: adenosine monophosphate (AMP), glycerophosphocholine (GPCh), hypoxanthine, L-aspartate, L-glutamate, L-methionine, L-tryptophan, myristoylcarnitine, and palmitoylcarnitine (PLC) (Fig. 4A). Among them, L-tryptophan (+0.749 fold), myristoylcarnitine (+0.913 fold), and PLC (+0.927 fold) were up-regulated in the SF group, while AMP (-0.750 fold), GPCh (-7.543 fold), hypoxanthine (-1.472 fold), L-aspartate (-2.202 fold), L-glutamate (-0.727 fold), and L-methionine (-5.456 fold) were significantly down-regulated in the SF group compared with the EC group. The summary profiles of the individual metabolites altered in the hippocampus of the 4-day and 15-day SF groups compared to the corresponding EC groups are shown in Table 1.

3.4 Metabolic pathway analysis

Because a statistical significance was observed only in the 15-day groups, metabolites identified from the 15-day SF experiments were used for metabolic interpretation by pathway analysis using MetPa analysis through MetaboAnalyst. As shown in Fig. 5, alanine, aspartate, and glutamate metabolism was determined as the key metabolism important in the 15-day SF group. In addition, metabolic pathways involved in other amino acid metabolism, aminoacyl-

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tRNA biosynthesis, and nucleotides were metabolic pathways that were significantly altered in the 15-day SF group.

4. Discussion

In the present untargeted metabolomics study, we found that alanine, aspartate, and glutamate metabolism pathway is the most altered metabolic pathway in the 15-day SF group compared to the 15-day EC group. The 4-day SF group showed a typical pattern of SF in the EEG and clearly differentiated in the metabolites profiles on PCA or PLS-DA compared to the corresponding EC group, but the identified metabolites were not statistically significant. Indeed, only the 4-day SF group showed a significant decrease in REM sleep compared to those in the baseline and corresponding EC group. Thus, it is not possible to exclude the possibility that the reduced REM sleep, rather than the SF itself, may have affected the distinction of metabolites in the 4-day groups.

Glutamate and aspartate are major excitatory neurotransmitters that can excite all neurons in the central nervous system including the hippocampal area (Fleck et al., 1993); however, excessive amounts of glutamate and aspartate can cause excitotoxicity through the NMDA receptor-dependent pathway. Glutamate level in hippocampi of sleep-restricted rats is likely to be affected by this type of SR and duration. In a study that examined glutamate level, 6 or 12 h of SD produced a greater increase in glutamate level in the rat hippocampus and thalamus compared with control groups (Cortese et al., 2010). Interestingly, in our study, these excitatory neurotransmitters (aspartate and glutamate) showed a temporal variation in the levels according to the SF duration. Levels of aspartate and glutamate showed a tendency to increase in the 4-day SF group (acute condition), but these were significantly decreased in the 15-day SF group (chronic condition), implying that lowering of excitatory neurotransmitter levels in the hippocampus of sleep-fragmented rats could be a protective mechanism against excitotoxicity induced by frequent arousal. This possibility might be partially supported by the findings from Novati et al. showing that 30 days of SR induced by a rotating drum attenuated the loss of cholinergic neurons in the nucleus basalis magnocellularis (NBM) when a neurotoxic dose of NMDA was administered into the NBM, indicating activation of a protective mechanism such as desensitization following SR against NMDA-mediated neurotoxicity (Novati et al., 2012). Whether these phenomena are specific

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events limited only to SF or can be generalized to any type of sleep disturbance requires further investigation. In addition to the role as a neurotransmitter, L-aspartate has a central effect on the stress responses. Intracerebroventricular injection of L-aspartate and its derivatives attenuated the stress response induced by social isolation (Erwan et al., 2012). To the best of our knowledge, aspartate level has not been determined in hippocampi of rats subjected to SF; however, the paradoxical SD (PSD) model using the multiple platform method, which also induces SF, 72-h of PSD increased aspartate in the rat hippocampus (Mohammed et al., 2011). Although not statistically significant, similar increases in aspartate levels were observed in the 4-day SF group. Therefore, it can be inferred that the stress response induced by SF in the hippocampus may show a temporal variation with the duration of SF and depend on the level of aspartate. To demonstrate this hypothesis, a functional study of the role of L-aspartate on the stress response in SF conditions is needed.

In the present study, up-regulation in tryptophan metabolism was observed in the 15-day SF group. The tryptophan level in the hippocampus increases under various stress conditions due to the increase in demand for 5-HT as a response to stress (Hery et al., 1970). In a previous study, increased 5-HT level was observed in the rat ventral hippocampal region following restriction of 21-days of rapid eye movement (REM) (da Silva Rocha-Lopes et al., 2017). This implies that increased tryptophan metabolism in the SF group can play a role in compensating for stress caused by frequent sleep interruption. In addition, tryptophan is a melatonin precursor, an important sleep-inducing hormone. However, the hippocampus is not a brain region directly associated with sleep induction; thus, the role of tryptophan as a sleep homeostatic regulator in rats subjected to SF is inconclusive. Melatonin also reduces the activity of the hippocampus and suppresses long-term potentiation induction (Ozcan et al., 2006; Wang et al., 2005).

PLC and Myristoylcarnitine belonging to long-chain acyl derivatives of carnitine also increased in the SF group. PLC can regulate the expression or activity of specific signaling molecules/apoptosis-related proteins/neurotransmission-related proteins (Mutomba et al., 2000; Sobiesiak-Mirska et al., 2003). PLC also plays an important role in the synthesis of lipids involved in neural membranes, brain plasticity, and downstream signal transduction (Jones et al., 2010). Currently, the biological function of myristoylcarnitine is not well known.

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Hypoxanthine is an important purine derivative related to the purine salvage pathway in the brain, a pathway to convert bases and nucleosides salvaged from dietary sources and tissue breakdown back into purine nucleotides. Although the reason for decreased hypoxanthine level in the SF group is unknown, one possible explanation is to maintain brain energy homeostasis imbalanced by frequent arousals. ATP is released from the synaptic terminal of neurons due to neuronal activity and released into extracellular space. Released ATP and adenosine, a degraded form of ATP, bind to purine type 2 receptors and to adenosine type 1 receptors expressed on glia (Fields and Burnstock, 2006), respectively, resulting in the release of pro-inflammatory cytokines such as tumor necrosis factor and interleukin-1β, thereby promoting sleep. Maintaining brain energy levels is considered the main function of sleep (Krueger et al., 2016; Scharf et al., 2008). Thus, the hypoxanthine level in the SF group may be reduced to compensate for energy consumption caused by increased neuronal activity through recycling of ATP.

We observed reduced AMP level in the SF group. AMP is a nucleotide used as a monomer in DNA and RNA and is composed of a phosphate group, sugar ribose, and adenine nucleobase. Decreases in AMP level were found in the Alzheimer mouse model (Esteve et al., 2017). AMP can exist in the form of a cyclic structure, cAMP. The principal target for cAMP is cAMP-dependent protein kinase A (PKA) whose activity is dependent on cellular levels of cAMP. It has been known that cAMP/PKA signaling has a pivotal role in hippocampus-dependent memory (Abel and Nguyen, 2008). Thus, a reduced AMP level might reflect a decrease in the reservoir that restores ATP levels and may reflect the functional attenuation of hippocampal-dependent memory induced by cAMP/PKA signaling.

L-methionine was also decreased in the hippocampus of rats subjected to chronic SF. According to a previous study, methionine level is likely associated with stress-induced cognitive impairments (Naninck et al., 2017). In that study, methionine level was decreased in brains of mice exposed to early-life stress (ES), and its supplementation restored methionine level and ES-induced cognitive impairments. In another study, long-term dietary restriction of methyl-donors including methionine, choline, and folic acid impaired hippocampus-dependent memory in mice, accompanying CpG hypermethylation of the specific gene promoter region in the hippocampus (Tomizawa et al., 2015). In addition,

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methionine is an acetylcholine precursor. Acetylcholinesterase (AChE) activity, a crucial enzyme that hydrolyzes acetylcholine, was increased in hippocampi of mice subjected to dietary restriction of methionine (Vucevic et al., 2016). Because methionine is an acetylcholine precursor and its deficiency resulted in increased activation of AChE, a decrease in methionine level in the hippocampus of rats subjected to SF may indicate the impaired status of cholinergic neurotransmission in hippocampus subjected to long-term SF.

GPCh showed the greatest reduction in the SF group. Choline is a precursor of acetylcholine, which is an important neurotransmitter for brain cognitive functions such as learning and memory (Fadda et al., 2000; Hasselmo, 2006). When choline intake is restricted due to a choline-deficient diet, AChE activity and (Na^+, K^+, and Mg^{2+}) ATPase activity were differentially activated in the hippocampus, hypothalamus, cerebellum, and pons (Liapi et al., 2009). These findings indicate the modulating roles of choline in cholinergic neurotransmission, neural excitability, and Mg^{2+} homeostasis in the brain. Whether GPCh has an important role by itself or as choline storage is unknown, but reductions of GPCh, similar to decreased methionine, likely reflect impaired cholinergic neurotransmission observed in the chronic SF group, possibly resulting in hippocampus-dependent learning and memory impairment.

Some limitations of our study should be addressed. First, we evaluated the metabolite profile of the whole hippocampus. It was therefore impossible to distinguish whether these compounds and amino acids were inside or outside the hippocampus cells. The analysis of metabolites from tissue samples can be influenced by mixing the intracellular and extracellular levels and the disturbance by intracellular enzymes which rapidly degrade the target substance. However, untargeted metabolomics of whole tissue sample are generally not intended to distinguish the inner and outer metabolites of the cell. An additional experiment, such as flux analysis using isotope labeled tracer, is needed to determine the origin of metabolites altered by SF. Second, there was no home cage control group in the present study. We used exercise group which offers the same amount of walking distance as the rats in the SF group, but continuous movement lasting 10 minute without a pause in the EC group may affect metabolite profiles of the hippocampus.

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In this untargeted metabolomics study, significantly altered metabolic pathways and metabolite profiles were observed in the hippocampi of rats subjected to chronic SF. The altered pathways and metabolite profiles varied according to the duration of SF. Further studies are required to clarify the biological meanings or functions of these metabolites in hippocampi of rats subjected to SF to understand the impact of SF on hippocampus-dependent neurocognitive behavior.

Author contributions statement

Conception and design, D.W.Y., H.N.K., C.H.Y., and C.S; data collection, D.W.Y., H.N.K., X.J., S.K.L., and J.K.K; data analysis and interpretation, D.W.Y., C.H.Y., H.N.K., and S.H.P; drafting the manuscript, D.W.Y., H.N.K., X.J., and C.H.Y; revision for important intellectual content, C.H.Y., C.S., and S.H.P.

Conflicts of interest

None of the authors have conflicts of interests to disclose.

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Figure legends

Figure 1. Schematic representation of the experimental protocols for SF and EC procedures, comparison of body weight, and effects of 4 days' and 15 days' SF or EC on sleep parameters.

A. Experimental protocol for the 4-day experiment. Prior to SF or EC, animals were placed in an individual wheel for 7 days to acclimate to the novel environment. B. Experimental protocol for the 15-day experiment. C. Average body weight (g) of 4-day group at baseline and on Day 4. D. Average body weight (g) of 15-day group at baseline and on Day 15. E. Comparison of time spent in Wake (%) between baseline and follow-up or between 4-day EC and SF groups (n=4, each). F. Comparison of time spent in NREM (%) between baseline and follow-up or between 4-day EC and SF groups. G. Comparison of time spent in REM (%) between baseline and follow-up or between 4-day EC and SF groups. H. Assessments of mean Wake bout number between baseline and follow-up or between 4-day EC and SF groups. I. Assessments of mean NREM bout number between baseline and follow-up or between 4-day EC and SF groups. J. Assessments of mean NREM bout length between baseline and follow-up or between 4-day EC and SF groups. K. Comparison of time spent in Wake (%) between baseline and follow-up or between 15-day EC and SF groups (n=4, each). L. Comparison of time spent in NREM (%) between baseline and follow-up or between 15-day EC and SF groups. M. Comparison of time spent in REM (%) between baseline and follow-up or between 15-day EC and SF groups. N. Assessments of mean Wake bout number between baseline and follow-up or between 15-day EC and SF groups. O. Assessments of mean NREM bout number between baseline and follow-up or between 15-day EC and SF groups. P. Assessments of mean NREM bout length between baseline and follow-up or between 15-day EC and SF groups. B, baseline; EC, exercise control; SF, sleep fragmentation; NREM, non-rapid eye movement; REM, rapid eye movement. $^{*}P<0.05$ vs. baseline, determined by paired *t*-test; $^{\dagger}P<0.05$ vs. corresponding EC group, determined by independent *t*-test.

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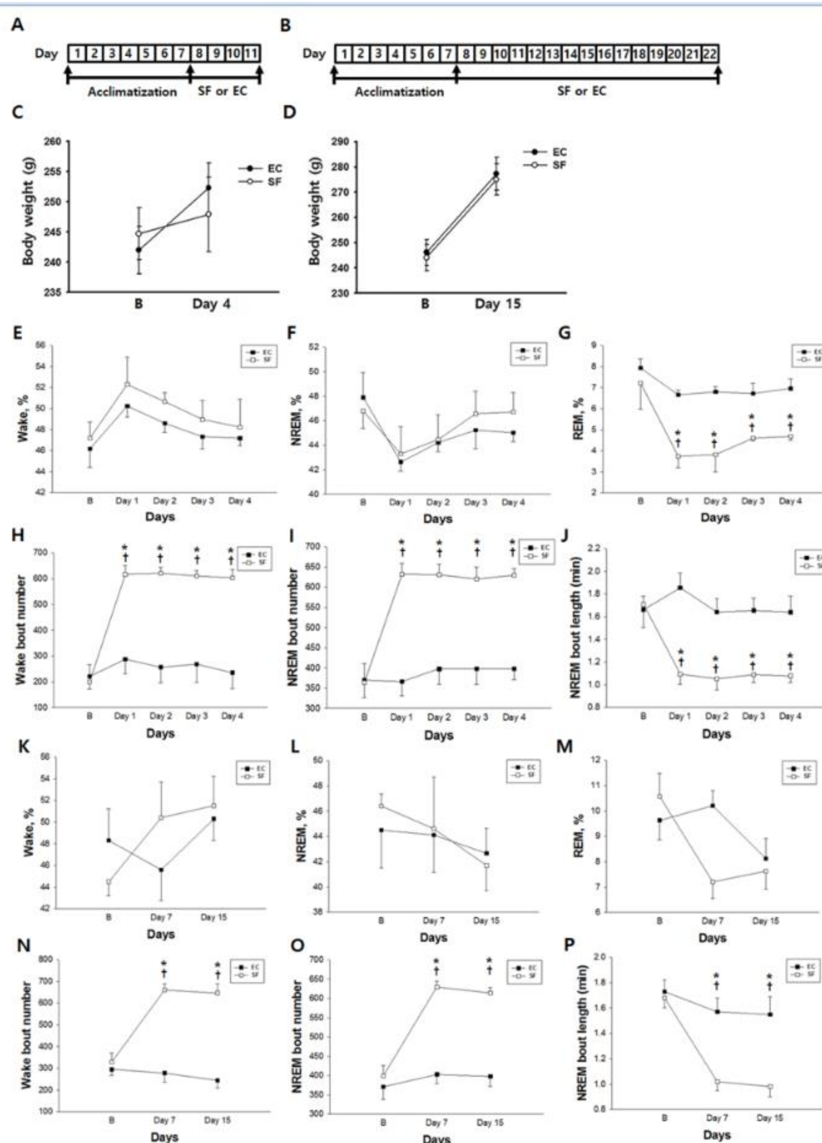

Figure 2. Difference in metabolite profiles between SF and EC groups.

Principle component analysis (PCA) and Partial least square discrimination analysis (PLS-DA) score plots of metabolite profiles from rat hippocampi in the SF and EC groups. PCA and PLS-DA score plots derived from untargeted metabolite profiling of hippocampus were analyzed by LC/MS. Each symbol represents the metabolomics profile of an individual sample. A. Comparison of SF and EC group from 4-day (black) and 15-day (red) by PCA. B. Comparison of SF (open boxes and triangles) and EC (squares and triangles) group from 4-day (black) and 15-day (red) by PLS-DA. C. Discrimination of metabolic profiles between 4-day SF (red triangles) and 4-day EC (black squares) groups by PLS-DA. D. Discrimination of metabolic profiles between 15-day SF (red triangles) and 15-day EC (black squares) groups

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by PLS-DA. SF, sleep fragmentation; EC, exercise control.

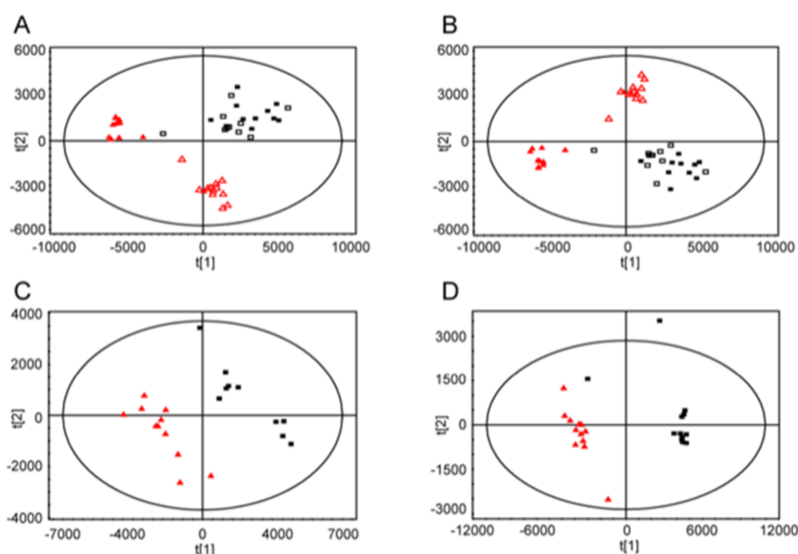

Figure 3. Differently expressed level of metabolites by SF

Differently expressed metabolites levels from 4-day (A) and 15-day (B) were presented including AMP, L-aspartate, L-glutamate, GPCh, hypoxanthine, jasmonic acid, L-methionine, myristoylcarnitine, PLC, and L-tryptophan. Y-axis represents relative intensity from LC/MS detection. SF, sleep fragmentation; EC, exercise control; PLC, palmitoylcarnitine; AMP, adenosine monophosphate; GPCh, glycerophosphocholine. *FDR adjusted $P<0.05$; **FDR adjusted $P<0.001$; ***FDR adjusted $P<0.00001$.

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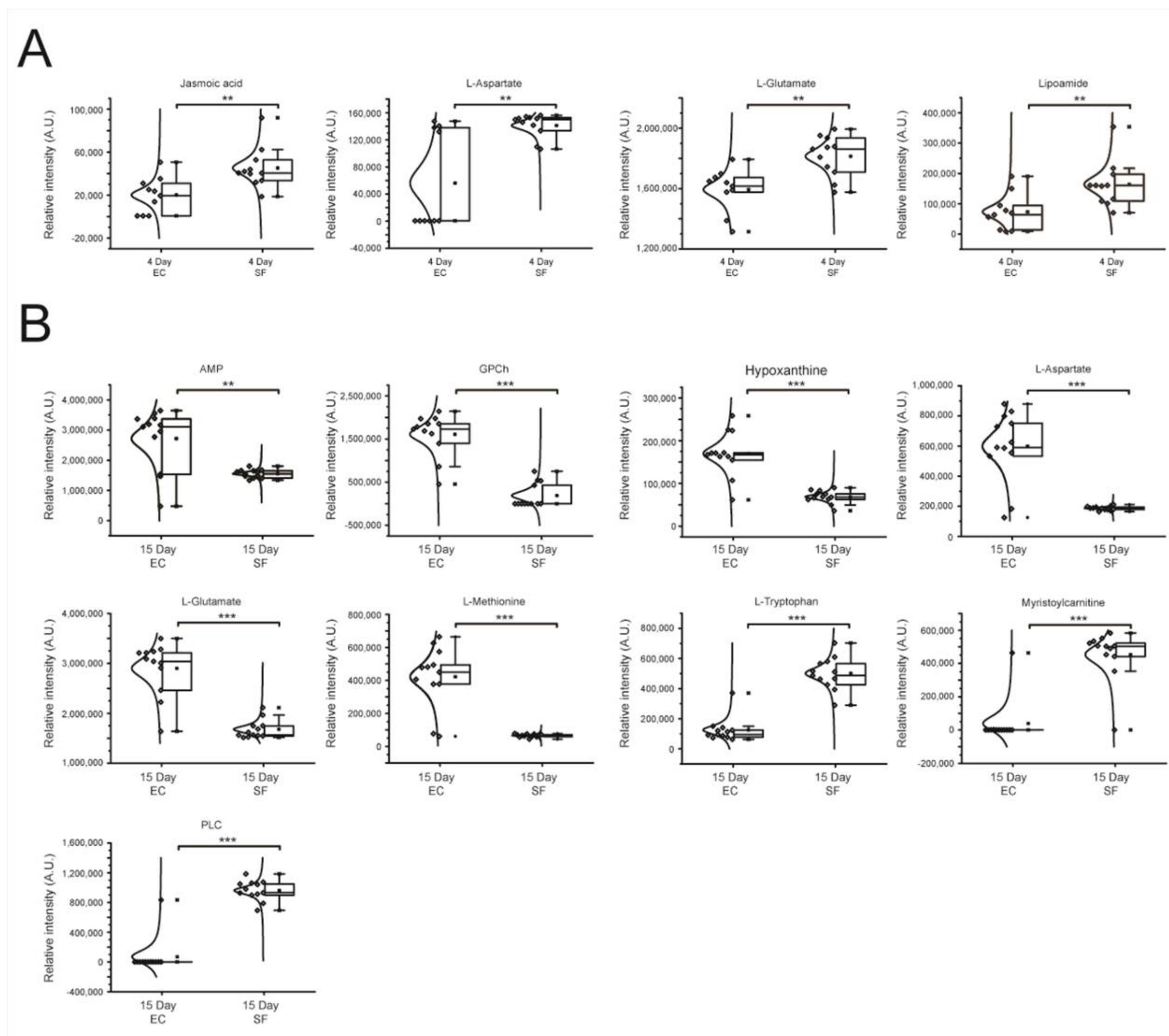

Figure 4. Identification of differentially expressed metabolites between SF and EC groups.

To identify differentially expressed metabolites between SF and EC groups, S-plot analysis was performed. Potential marker signals that were significantly biased across the two groups are enclosed in red-color boxes. A. Differentiation of contributing signals between 4-day SF and 4-day EC groups. B. Differentiation of contributing signals between 15-day SF and 15-day EC groups. The upper right panel shows contributing metabolites in the SF group and the lower left panel in the EC group. L-Asp, L-aspartate; L-Trp, L-tryptophan; AMP, adenosine monophosphate; L-Glu, L-glutamate; L-Met, L-methionine.

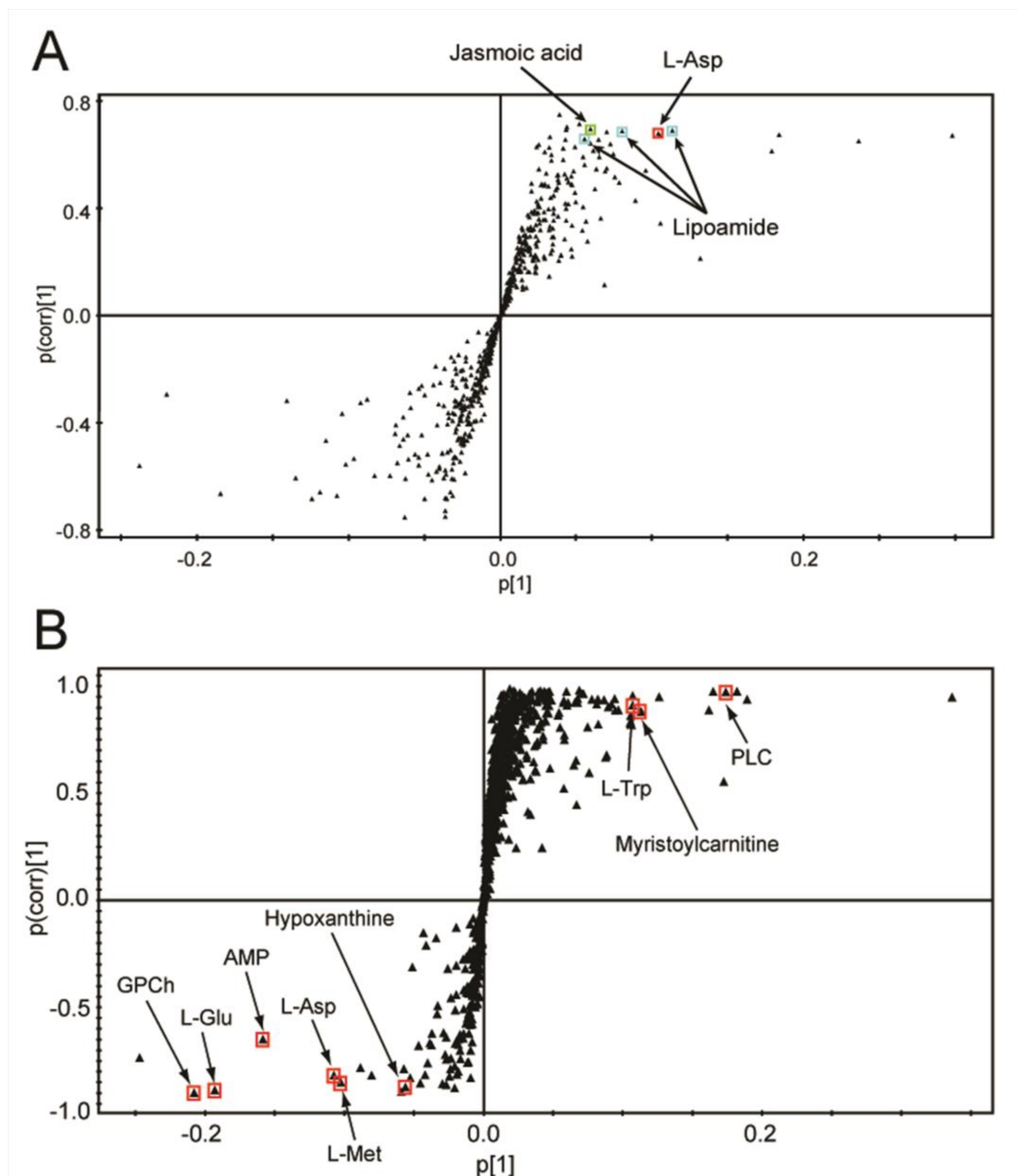

Figure 5. The analysis results of metabolic pathways significantly altered in the 15-day SF group.

MetPa analysis was performed to determine metabolic pathways affected by SF. Metabolites from comparison between 15-day SF and 15-day EC groups were used to draw pathway contributions using MetPa analysis. The pathway impact values on X-axis were results from pathway topology analysis and y-axis values present a view of all the matched pathways arranged by p-values (pathway enrichment analysis). Each different radius sizes indicates the number of metabolites matched on the pathway and colors from beige to red is matched with p-value from pathway enrichment analysis. Therefore, alanine, aspartate, and glutamate

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metabolism is the most important pathway. 1, Alanine, aspartate, and glutamate metabolism; 2, Aminoacyl-tRNA biosynthesis; 3, Nitrogen metabolism; 4, D-glutamine and D-glutamate metabolism; 5, Purine metabolism; 6, Arginine and proline metabolism; 7, Cysteine and methionine metabolism; 8, Histidine metabolism; 9, Glycine, serine and threonine metabolism; 10; Tryptophan metabolism.

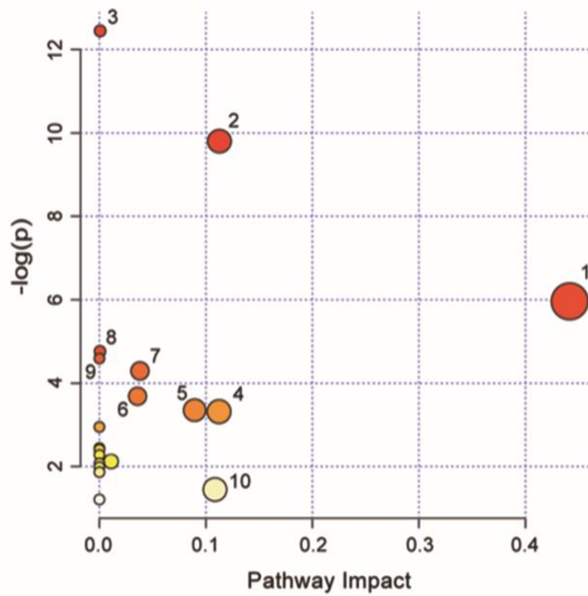

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Table

Table 1. Metabolite profiles identified in the 4-day SF and 15-day SF groups based on LC/MS analysis

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Table 1. Metabolite profiles identified in the 4-day SF and 15-day SF groups based on LC/MS analysis

4-day SF					
Identification	Measured m/z	RT (min)	*P*-value	FDR adjusted *P*-value	Fold change compared with EC
Jasmonic acid	211.17522	14.68	4.674E-03	0.0511	0.556
L-aspartate	134.08177	3.39	4.494E-03	0.0511	0.602
L-glutamate	148.08522	2.74	1.807E-03	0.0683	0.121
Lipoamide	189.1447	14.67	7.246E-03	0.0511	0.553
15-day SF					
Metabolites	Measured m/z	RT (min)	*P*-value	FDR adjusted *P*-value	Fold change compared with EC
AMP	348.21896	2.83	1.972E-03	1.577E-04	- 0.750
GPCh	258.22021	2.72	1.552E-08	2.021E-07	- 7.543
Hypoxanthine	137.10307	4.11	2.250E-05	4.812E-07	- 1.472
L-aspartate	134.08177	3.39	8.481E-05	9.110E-06	- 2.202
L-glutamate	148.08522	2.74	3.298E-06	2.303E-06	- 0.727
L-methionine	150.12025	3.33	3.942E-05	2.330E-06	- 5.456
L-tryptophan	205.16923	8.74	2.497E-09	8.629E-09	0.749
Myristoylcarnitine	372.5415	26.64	4.689E-07	1.118E-05	0.913
PLC	400.58891	27.18	1.426E-10	2.300E-08	0.927

SF, sleep fragmentation; EC, exercise control; LC/MS, liquid chromatography/mass spectrometry; m/z, mass-to-charge ratio; RT, retention time; PLC, palmitoylcarnitine; AMP, adenosine monophosphate; GPCh, glycerophosphocholine

The fold change values are from area-normalized peak intensities and calculated by following formula: (SF-EC)/SF. Negative values represent decreases and positive values represent increases. All the LC/MS-identified metabolites with significant changes are included. *P*-values and the FDR adjusted *P*-value were used for metabolites selection.

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