

## Changes in brain metabolites related to stress resilience: Metabolomic analysis of the hippocampus in a rat model of depression

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### ABSTRACT

The ability to cope successfully with stress is known as ‘resilience’, and those with resilience are not prone to developing depression. One preclinical animal model for depression is the chronic mild stress (CMS) model. There are CMS-resilient (do not manifest anhedonia) and CMS-susceptible (manifest anhedonia) rats. This study aimed to investigate the differences in the profiles of hippocampal metabolites between susceptible and resilient rats, and to identify a biomarker that can distinguish the two. We divided stress-loaded rats into susceptible and resilient types based on their sucrose preference values. We then conducted brain-derived neurotrophic factor (BDNF) quantification and metabolomic analysis in the hippocampus. Compared to the controls, no significant differences were observed in the hippocampal BDNF levels of susceptible and resilient rats. However, the control rats were clearly distinguishable from the susceptible rats in terms of their brain metabolite profiles; the control rats were difficult to distinguish from the resilient rats. CMS model rats showed an increase in the levels of N-acetylaspartate and glutamate, and a decrease in the levels of aspartate and  $\gamma$ -aminobutyric acid in the hippocampus. Of the 12 metabolites measured in the present study, N-acetylaspartate was the only one that could differentiate the three types (control, susceptible, and resilient) of rats. Thus, brain metabolomic analyses can not only distinguish CMS model rats from control rats, but also indicate stress susceptibility. The variation in the levels of N-acetylaspartate in the hippocampus of control, resilient, and susceptible rats demonstrated that it could be a biomarker for stress susceptibility.

### 1. Introduction

The World Health Organization announced in March 2018 that the number of patients afflicted with depression has reached 300 million globally [1]. Depression presents as a variety of symptoms including insomnia, weight loss, fatigue, and loss of concentration, with depressed mood and loss of interest or pleasure (anhedonia) being positioned as the core symptoms of depression [2]. Stressful life events such as becoming a victim of crime, financial problems, and divorce are risk factors for depression [3]. However, not everyone who has experienced stressful life events develops depression; some people adapt to stress. The ability to successfully adapt to stress is termed ‘resilience’ [4], and individuals who have acquired resilience are believed to have active coping capabilities towards stress [5].

A chronic mild stress (CMS) model created by chronic stress was reported to have favourable construct, face, and predictive validity [6]; additionally, chronic stress is known to induce anhedonia, which is a core symptom of depression [7]. In the CMS models, anhedonia has been evaluated by a behavioural test known as the sucrose preference test. However, CMS animal models require several weeks to be created; they are also known to have poor reliability [8]. We previously confirmed that rats chronically loaded with stress experience a reduction in sucrose preference (i.e., they present symptoms of anhedonia) [9]. However, not all rats exposed to stress exhibit a reduction in sucrose preference [9,10]. This mixture of types is believed to underlie the poor reliability of the animal model of depression created via chronic stress. In addition, in CMS model rats, those that present with anhedonia are said to be susceptible individuals, and those that do not are said to be

*Abbreviations:* CMS, chronic mild stress; BDNF, brain-derived neurotrophic factor; NMR, nuclear magnetic resonance; PLS-DA, partial least squares discriminant analysis; CI, confidence interval; VIP, variable importance in the projection; MDD, major depressive disorders; TrkB, tropomyosin-related kinase B; GABA,  $\gamma$ -aminobutyric acid; GAD, glutamate decarboxylase

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resilient rats. These rats are used in studies on stress resilience [11,12]. Furthermore, it has been reported that responses to antidepressants differ between low- and high-responders to stress [13,14,15]. It is therefore important to adjust the behavioural characteristics of individual animals at a stage prior to stress loading.

A number of studies state that the hippocampus is involved not only in memory and cognitive function, but also in mood regulation [16,17,18]. Moreover, the hippocampus' structure and function are highly sensitive to stress [19,20]. Chronic stress is known to reduce the level of brain-derived neurotrophic factor (BDNF), which is involved in neuronal survival and synaptic plasticity within the hippocampus [21]. In addition, intrahippocampal injection of BDNF produces antidepressant-like effects. BDNF is also necessary for the effects of some antidepressants [22,23]. A number of basic studies have demonstrated that the concentration of BDNF in the hippocampus can be used as a marker for a compound's anti-depressive activity [24,25].

We have already found that profiles of brain metabolites of CMS model rats can be clearly distinguished using high resolution magic angle spinning <sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy, and reported that the metabolomic analysis can assess the stressed state [9]. Based on our previous results, we assumed the poor reliability of CMS models to be attributable to the intermixing of resilient rats with susceptible rats. Therefore, in the current study, we opted to use resilient and susceptible rats that had undergone chronic loading of stress to perform a metabolomic analysis of the rats' hippocampal tissues, and to investigate the differences in the profiles of brain metabolites between the resilient and susceptible states. We also aimed to identify biomarkers that can distinguish between resilient and susceptible rats.

## 2. Materials and methods

### 2.1. Animals

We purchased 39 7-week-old male Wistar/ST rats from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). The rats were acclimatized for a minimum of one week at 25 ± 2 °C, relative humidity of 55 ± 5%, with a 12-h light-dark cycle (7:00–19:00), and with *ad libitum* food (Lab MR Stock; Nosan Corp., Kanagawa, Japan) and water intake. Of the 39 rats, 21 were randomly allocated to the Stressed Group, and the remaining 18 were allocated to the unstressed Control Group.

All animal experiments were performed in accordance with the Guideline for Proper Conduct of Animal Experiments, established by the Science Council of Japan after approval by the Institutional Animal Care Committee of Josai University (approval number: H29102-2017/10/5).

### 2.2. Establishment of chronic stress model rats

Chronic stress was implemented in accordance with previously reported methods [9]. Fig. 1 presents the schedule of stress loading. Rats in the Stressed Group were loaded with two types of stress (a combination of long-term stressors [ > 12 h] and short-term stressors [ ≤ 1 h], respectively) for 4 weeks (Days 2–29). Long-term stressors included food and water deprivation (24 h), cage tilt (21 h, 45°), light during the dark cycle (12 h), soiled cage (24 h), and water deprivation (conducted for 24 h followed by 1-h empty bottle; total 25 h). Short-term stressors included foot shock (1.5 mA, 15 s per stimulation with 60 s intervals, repeated eight times), forced swimming (5 min at 4 °C), tail pinching (1 min), and white noise (85 dB, 15 s per stimulation with 15 s intervals, repeated 120 times).

### 2.3. Body weight and behavioural testing

We performed body weight measurement and two types of behavioural tests: sucrose preference and open field testing. The details are provided below.

- (1) Body weight: Measured between 9:00 and 9:30 once every 7 days from Day 0 (pre-experiment), namely Days 7, 14, 21, and 28.
- (2) Sucrose preference test: Conducted once every 7 days from Day 1 (pre-experiment), namely Days 8, 15, 22, and 29 [26]. A training session to acclimatize the rats to the sucrose solution was conducted 72 h prior to the initial preference test, in which two bottles of 1% sucrose solution were placed in each cage, and 24 h later the 1% sucrose in one bottle was replaced with tap water for a further 24 h. After acclimatization, the rats were deprived of food and water for 24 h, and then underwent a 24-hour sucrose preference test (Day 1). The sucrose preference was calculated as a percentage of the consumed 1% sucrose solution relative to the total volume of liquid intake.

It was suggested that stress response varied for each individual according to the behaviour of individual rats during the pre-experiment stage. [27]. Therefore, to align the sucrose preference between the Stressed Group and the Control Group, we excluded rats whose sucrose preference in the pre-experiment was < 80.0%.

Sucrose preference values are used to determine whether a rat is susceptible or resilient to stress [11,12]. After the completion of a 4-week stress load, rats in the Stressed Group whose sucrose preference on Day 29 was < 65% were classified into the Susceptible Group, and the remaining rats in the Stressed Group whose preference changes from the pre-experiment stage were < 10% were classified into the Resilient Group.

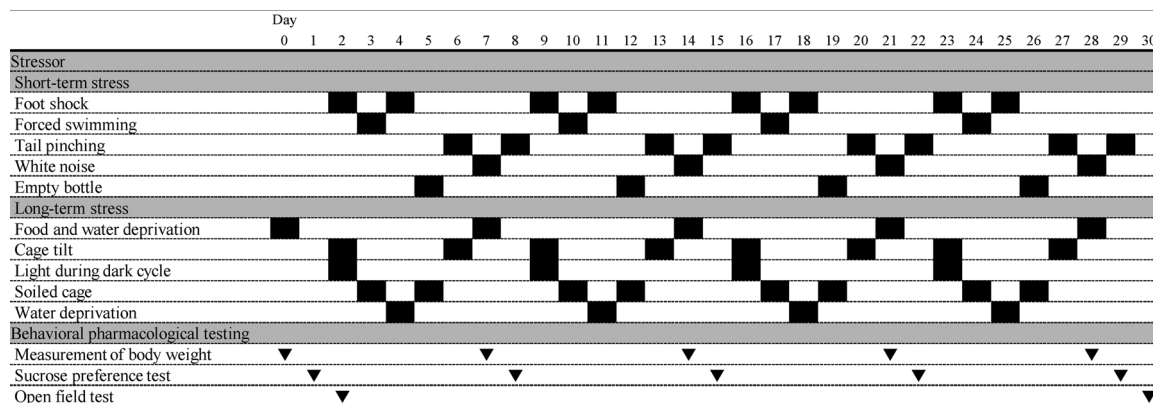


Fig. 1. Stress loading schedule.

Day 0 to Day 2 is the pre-experiment period. The stress loading period is from after implementation of the open field test (Day 2) to Day 29.

(3) Open field test: In an open field test, rats are allowed to move around and act freely in a quiet environment. We implemented this test on Day 2 (pre-experiment) and Day 30, in accordance with previously reported methods [28]. 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 centre of the apparatus and its behaviour was video-recorded for 6 min. The occurrences of grid line crossings, rearing, defecation, and grooming in the recorded video (5 min; min 1–6) were counted to identify locomotor activities and anxiety-like behaviours [29,30]. Open field was scored by hand by observers blinded to the stress groups.

#### 2.4. Sample preparation

After the final open field test, we decapitated the rats under anaesthesia (50 mg/kg of pentobarbital sodium). After rapidly removing the brain tissue, we separated the brain along the longitudinal cerebral fissure into right and left hemispheres. The hippocampus in the right and left brains, respectively, were dissected and quick-frozen using liquid nitrogen, then stored at  $-80^{\circ}\text{C}$  until the measurement dates. To ensure constancy of the post-mortem changes in the brain metabolites, we made sure to complete the entire set of operations within 10–15 min.

#### 2.5. Measurement of mature BDNF in the hippocampus

The hippocampus of the left hemisphere that had been frozen was thawed under ice-cooling, placed inside a Teflon homogenizer, and had the following items added per 10 mg of wet weight: 100  $\mu\text{L}$  of ice-cold RIPA buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50  $\mu\text{M}$  bestatin, 10  $\mu\text{M}$  pepstatin A, 20  $\mu\text{M}$  leupeptin hemisulphate, 0.8  $\mu\text{M}$  aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM ethylenediamine tetraacetic acid. After homogenizing under ice-cooling for 5 min at 200 rpm, the homogenate was centrifuged at  $4^{\circ}\text{C}$ ,  $14,000 \times g$ , for 30 min. The supernatant was then collected. After diluting the supernatant 500-fold in ice-cold RIPA buffer, we followed the manufacturer's instructions and quantified the mature BDNF present in the hippocampal tissue using the Mature BDNF, Human/Mouse/Rat, Rapid ELISA Kit (Biosensis Pty Ltd., Australia). The standards and samples were run in duplicate.

Protein quantification was performed using a bicinchoninic acid assay (BCA protein assay kit-reducing agent compatible, Thermo Scientific, MA, USA).

#### 2.6. Measurement of the NMR spectra

We used a Varian INOVA-700 NMR instrument at 699.7 MHz as the  $^1\text{H}$  frequency, equipped with a FASTNANO™ probe head (Agilent Technologies, Santa Clara, CA, USA). To allow detection, 43  $\mu\text{L}$  deuterated water containing 2.5 mM sodium-3-(trimethylsilyl)-1-propane-1,1,2,2,3,3-d 6-sulfonate was added to each hippocampus from the right hemisphere (wet weight: 10–20 mg). The sample was manually homogenized with 20 rotations of a polypropylene pestle in a micro-tube. After the entire volume of the brain homogenate was transferred into a 43- $\mu\text{L}$  glass cell with a Pasteur pipette, the cell was set into a 4 mm outer diameter zirconium oxide rotor. This process was repeated for each hippocampal sample. The rotors were then loaded into the NMR spectrometer.

The parameters of the NMR spectrometer were as follows:  $90^{\circ}$  pulse width, 7.50–7.75  $\mu\text{s}$  (measured and set for each sample); relaxation delay, 2.000 s; number of data points, 32k complex; observation width, 8389.3 Hz; number of scans, 128; and rotation speed, 5000 Hz A pre-saturation sequence was performed to reduce water-derived signals. To reduce broad signals derived from macromolecules such as proteins,

and to enable the conductance of subsequent analyses using small molecules, we measured the NMR spectrum using the Carr-Purcell-Meiboom-Gill spin-echo pulse sequence ( $[\text{D}-90^{\circ}-(180^{\circ})\text{-n-FID}]$ , where FID is free induction decay) with a fixed spin-spin relaxation delay,  $2\pi\tau$ , of 60 ms [ $\tau = 400\ \mu\text{s}$ ]. Steps were taken to ensure consistency of the changes in metabolites among the samples during measurements, and the measurement temperature was maintained at 298 K. This operation was carried out using VnmrJ software (Ver. 4.0; Agilent Technologies).

#### 2.7. Processing of the NMR spectra

After Fourier transformation of all acquired free induction decays, we manually performed phase and baseline corrections using 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 [31,32].

For multivariate analysis of NMR-derived data, all 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: 4.60–4.92 ppm) in the acquired NMR spectra was integrated in 0.04 ppm buckets to obtain 239 variables. Each bucket was then normalized to yield a total integrated area of 100.

#### 2.8. Statistical analysis

A two-tailed Student's *t*-test was performed to compare the results of body weight measurement and behavioural testing and the amount of BDNF contained in the hippocampal tissues between the Control Group and the Stressed Group. When performing a three-group comparison among the Control Group, the Resilient Group, and the Susceptible Group, one-way ANOVA or two-way repeated measures ANOVA followed by a multiple comparison using Tukey-Kramer method was conducted. We also subjected each of the NMR spectra buckets to the Student's *t*-test to identify relative changes in each brain metabolite. To implement a two-tailed Student's *t*-test, ANOVA, and a multiple comparison, we used R software (version 3.2.2, R Foundation for Statistical Computing, Vienna, Austria) for Windows®.

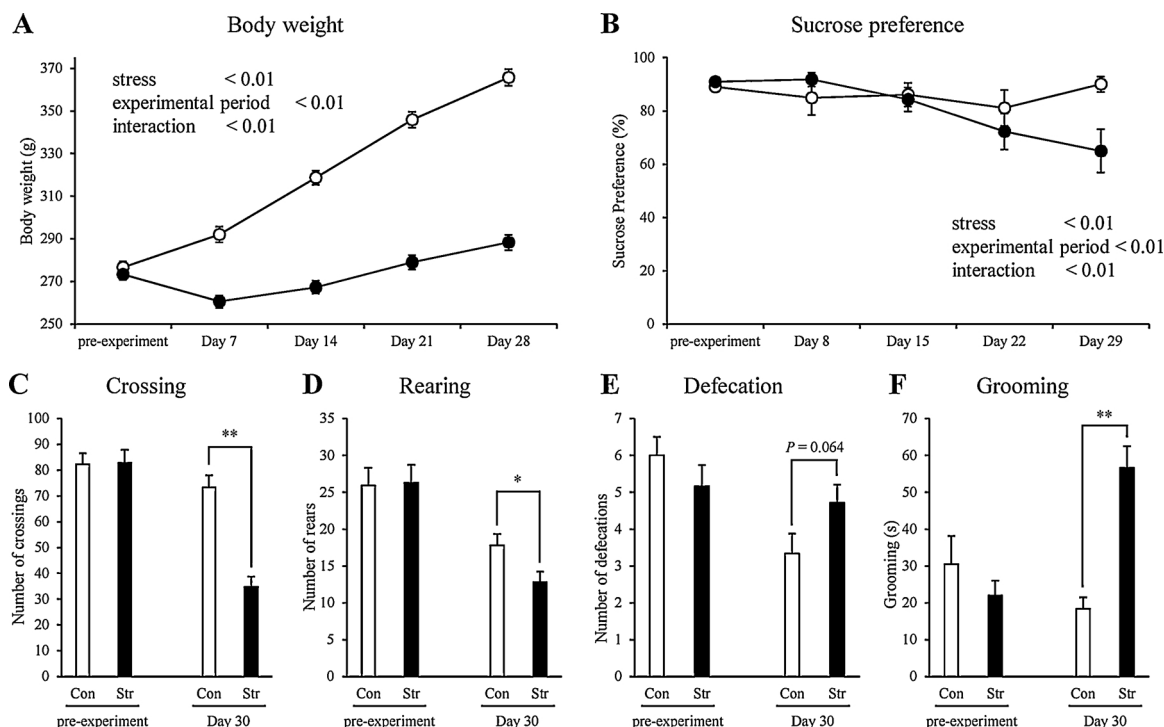
To identify the differences in brain metabolite profiles, we conducted a partial least squares discriminant analysis (PLS-DA), using SIMCA-P multivariate analysis software (version 13.0.3, Umetrics, Umea, Sweden). To avoid overfitting to the PLS-DA model, we conducted a permutation test with 100 iterations [33].

### 3. Results

#### 3.1. Establishment of CMS model rats

The results of the pre-experiment sucrose preference test indicated that the sucrose preference exceeded 80.0% in 14 of the 18 rats in the Control Group, and exceeded 80.0% in 18 of the 21 rats in the Stressed Group. Fig. 2 presents the results of body weight and behavioural testing in the pre-experiments and after 4 weeks of stress loading in the Control Group ( $n = 14$ ) and the Stressed Group ( $n = 18$ ).

A repeated measures ANOVA on Body Weight (Fig. 2A) showed that the presence of stress [ $F_{(1,120)} = 3668.5$ ,  $P < 0.01$ ] and the experiment duration [ $F_{(4,120)} = 634.08$ ,  $P < 0.01$ ] significantly influenced body weight. Moreover, a statistical interaction was observed between the presence or absence of stress and the experiment duration [ $F_{(4,120)} = 303.64$ ,  $P < 0.01$ ]. Likewise, with respect to sucrose preference (Fig. 2B), presence or absence of stress [ $F_{(1,120)} = 12.446$ ,  $P < 0.01$ ] and the experiment duration [ $F_{(4,120)} = 7.171$ ,  $P < 0.01$ ] significantly influenced sucrose preference. A statistical interaction was observed between the presence of stress and the experiment duration



**Fig. 2.** Influence of stress load on rats' behaviours and actions. Following two-way repeated measures ANOVA on body weight (A) and sucrose preference (B), the two-tailed student's *t*-test was performed for each time point. Regarding crossing (C), rearing (D), defecation (E), and grooming (F), the two-tailed student's *t*-test was performed for the pre-experiment period and Day 30. All data are presented as mean ± SEM. \*: *P* < 0.05, \*\*: *P* < 0.01. Con: Control Group (n = 14); Str: Stressed Group (n = 18).

[ $F_{(4,120)} = 6.013, P < 0.01$ ].

In the pre-experiments, no significant differences were observed between the Control Group and the Stressed Group in any of the six evaluation items (sucrose preference, body weight, crossing, rearing, defecation, and grooming), confirming that the behavioural features were the same between the two groups (Fig. 2A–F).

While body weight decreased significantly immediately after stress loading [Day 7:  $t_{(30)} = 6.62, P < 0.01$ ; Fig. 2A], sucrose preference decreased significantly 4 weeks after starting the stress loading [ $t_{(30)} = 2.94, P < 0.05$ ; Fig. 2B]. Crossing and rearing, both of which are indicators of locomotor activity, decreased significantly due to stress loading [ $t_{(30)} = 6.01, P < 0.01$  and  $t_{(30)} = 2.54, P < 0.05$ , respectively; Fig. 2C and D]. Defecation, an indicator of anxiety, exhibited a tendency to increase [ $t_{(30)} = -1.74, P = 0.064$ ; Fig. 2E], while grooming, which is yet another indicator of anxiety, exhibited a significant increase [ $t_{(30)} = -5.20, P < 0.01$ ; Fig. 2F]. Based on the above, CMS model animals were created by chronic stress.

### 3.2. Detection of resilient rats based on sucrose preference values

Fig. 3 presents the distribution of sucrose preferences that were measured every week from the pre-experiment period.

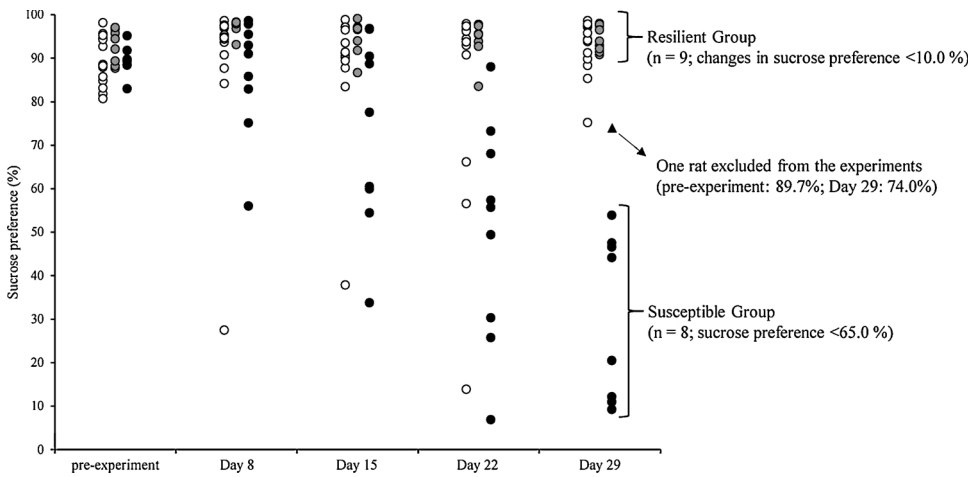
The sucrose preference of each rat in the Control Group, indicated by a white circle, decreased in some rats. However, the majority of the rats exhibited almost no changes in sucrose preference throughout the 4-week period. The sucrose preference of rats in the Stressed Group, indicated by either a grey or a black circle, was distributed between 80% and 100% at the pre-experiment stage. However, after stress loading, the rats in the Stressed Group were clearly divided into those whose sucrose preference decreased with time (indicated by a black circle) and those whose sucrose preference remained unchanged (indicated by a grey circle). In our study, in line with previously reported literature [12], the rats in the Stressed Group (n = 18, grey and black circles, Fig. 3) whose sucrose preference on Day 29 was < 65% were

classified into the Susceptible Group (n = 8, black circle); of the remaining rats in the Stressed Group, those whose change in sucrose preference was < 10% were classified into the Resilient Group (n = 9, grey circle). However, one rat in the Stressed Group had a sucrose preference of 74.0% as of Day 29 (Fig. 3; excluded). Because this rat did not belong to either the Resilient Group or the Susceptible Group, it was excluded from subsequent experiments.

After the Stressed Group had been divided into Resilient and Susceptible Groups, the results of body weight and behavioural testing were compared among the three groups: namely, the Control, Resilient, and Susceptible Groups. The findings are presented in Fig. 4. Repeated measures ANOVA on Body Weight (Fig. 4A) demonstrated that the group [ $F_{(2,112)} = 1737.33, P < 0.01$ ] and the experiment duration [ $F_{(4,112)} = 622.91, P < 0.01$ ] significantly influenced body weight. Further, a statistical interaction was found between the group and the experiment duration [ $F_{(8,112)} = 141.68, P < 0.01$ ].

One-way ANOVA on crossing, rearing, defecation, and grooming in the pre-experiment stage demonstrated no significant difference in any of the surveyed items: crossing [ $F_{(2,28)} = 0.12, P = 0.89$ ], rearing [ $F_{(2,28)} = 0.50, P = 0.61$ ], defecation [ $F_{(2,28)} = 0.42, P = 0.66$ ], and grooming [ $F_{(2,28)} = 0.56, P = 0.58$ ]. Therefore, at the pre-experiment stage, no significant differences were observed among the Control, Resilient, or Susceptible Groups, confirming that the behavioural features were the same among the three groups (Fig. 4A–E).

One-way ANOVA on evaluation items of the open field test following the completion of 4-week stress load indicated significant differences amongst three groups in crossing [ $F_{(2,28)} = 16.16, P < 0.01$ ], rearing [ $F_{(2,28)} = 4.21, P < 0.05$ ], and grooming [ $F_{(2,28)} = 12.09, P < 0.01$ ]. However, no significant differences were detected in defecation [ $F_{(2,28)} = 1.39, P = 0.27$ ]. Subsequently, a multiple comparison was conducted using Tukey-Kramer method. The crossing of the Resilient Group and the Susceptible Group decreased significantly compared to that of the Control Group (test statistic = 4.67, *P* < 0.01 and test statistic = 4.77, *P* < 0.01, respectively). However, no



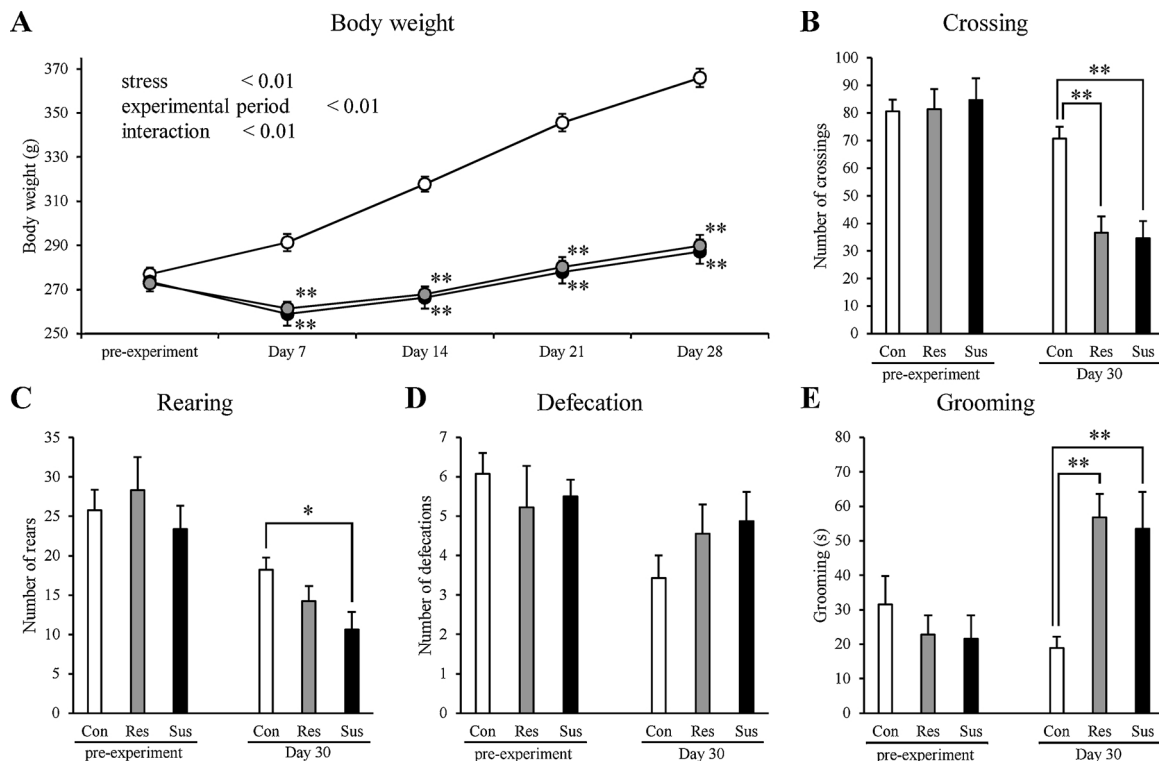
**Fig. 3.** Timeline changes in sucrose preference of individual rats. The white circle indicates Control rats, and either grey or black circles indicate rats in the Stressed Group. The grey and black circles represent resilient rats and susceptible rats, respectively. One rat in the Stressed Group whose sucrose preference was 74.0% on Day 29 was excluded from the experiments.

significant differences were observed between the Resilient Group and the Susceptible Group (Fig. 4B). Rearing decreased significantly in the Susceptible Group compared to the Control Group (test statistic = 2.85,  $P < 0.05$ ), but no significant differences were observed between the Resilient Group and the Control Group (Fig. 4C). No significant differences were seen in defecation among three groups (Fig. 4D). Grooming in the Resilient Group and the Susceptible Group increased significantly compared to that of the Control Group (test statistic = 4.32,  $P < 0.01$  and test statistic = 3.81,  $P < 0.01$ ), but no significant differences were observed between the Resilient Group and the Susceptible Group (Fig. 4E).

3.3. Amount of mature BDNF in the hippocampal tissues

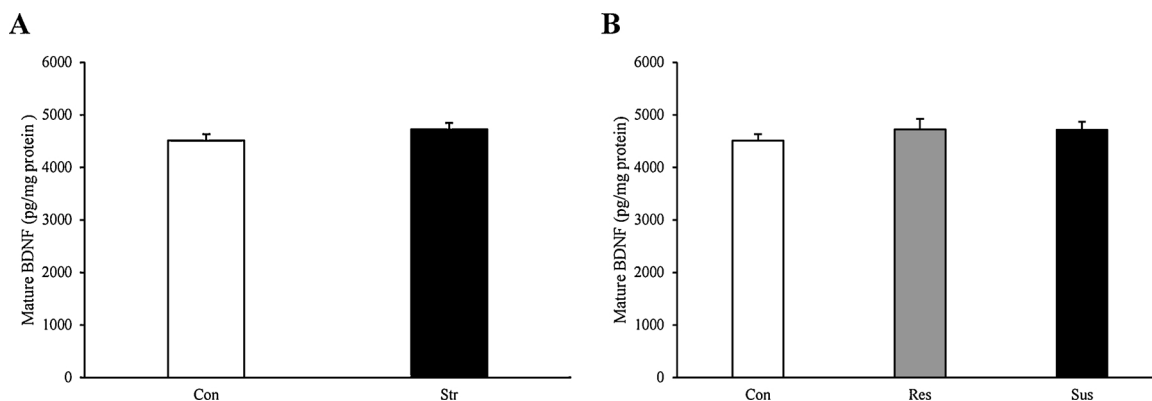
Fig. 5 indicates the amount of mature BDNF in each group. The amount of mature BDNF in the hippocampus was  $4,507.6 \pm 123.0$  pg/mg protein (mean  $\pm$  SEM) in the control group and  $4,722.9 \pm 124.0$  pg/mg protein in the Stressed Group, with no significant difference between the two groups [ $t_{(29)} = -1.22$ ,  $P = 0.23$ ; Fig. 5A].

The Stressed Group was further divided into the Resilient Group and the Susceptible Group, and one-way ANOVA was carried out on three groups: Control, Resilient and Susceptible. However, no significance was detected. The multiple comparison also revealed no significant differences in the hippocampal BDNF among three groups. (Control Group =



**Fig. 4.** Changes in behaviours related to sensitivity to stress.

White circles represent the Control Group, grey circles represent the Resilient Group, and black circles represent the Susceptible Group. Following two-way repeated measures ANOVA on body weight (A), a multiple comparison was conducted for each time point using Tukey-Kramer method. Regarding crossing (C), rearing (D), defecation (E), and grooming (F), one-way ANOVA was performed for the pre-experiment period and Day 30, followed by a multiple comparison using Tukey-Kramer method. All data are presented as mean  $\pm$  SEM. \*:  $P < 0.05$  compared to the Control Group, \*\*:  $P < 0.01$  compared to the Control Group. Con: Control Group (n = 14); Res: Resilient Group (n = 9); Sus: Susceptible Group (n = 8).



**Fig. 5.** Comparison of the amount of mature BDNF in the hippocampal tissue. Two-tailed student's *t*-test was conducted to compare two groups (A). For comparison among 3 groups (B), one-way ANOVA was performed followed by a multiple comparison using Tukey-Kramer method. All data are presented as mean ± SEM. Con: Control Group (n = 14); Str: Stressed Group (n = 18); Res: Resilient Group (n = 9); Sus: Susceptible Group (n = 8); BDNF: brain-derived neurotrophic factor.

4,507.6 ± 123.0 pg/mg protein, Resilient Group = 4,725.6 ± 200.8 pg/mg protein, and Susceptible Group = 4,719.9 ± 151.4 pg/mg protein; Fig. 5B).

### 3.4. Profiles of the brain metabolites of resilient and susceptible rats

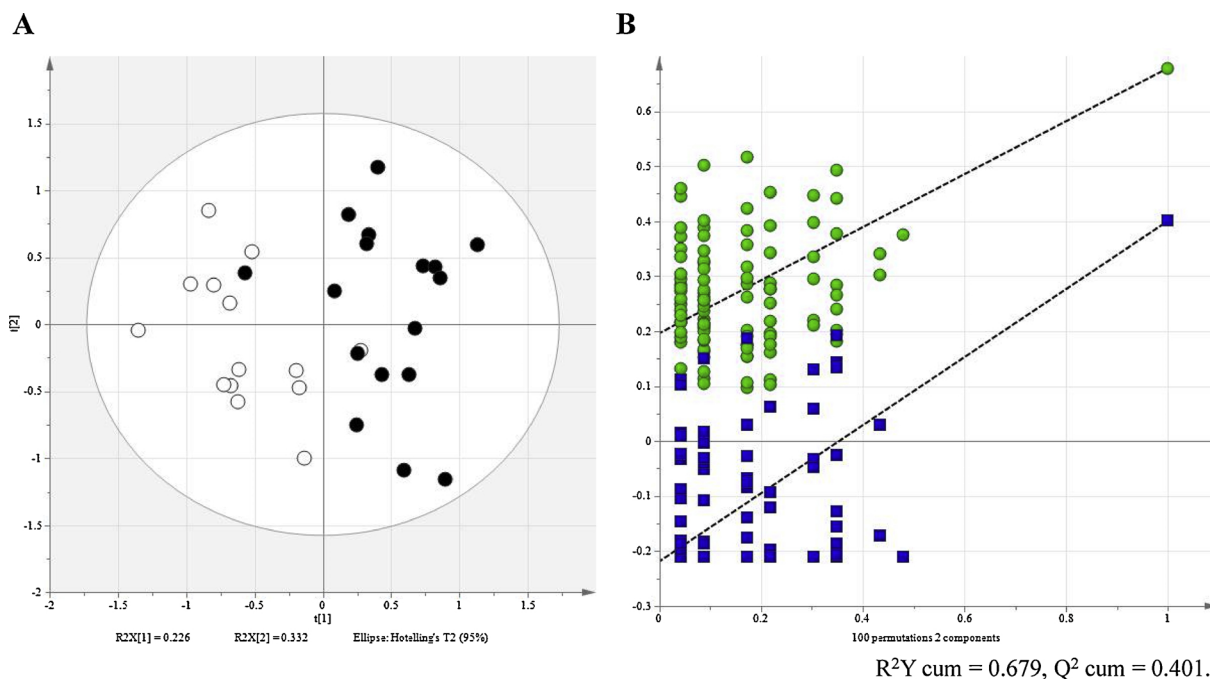
Fig. 6 provides the profiles of the brain metabolites of the Control Group and the Stressed Group, as well as their validity plot (permutation plot). We performed a PLS-DA on the NMR spectra's bucket integral value and found that the brain metabolite profiles were distinguishable between the Control Group (white circle) and the Stressed Group (black circle). However, the two groups could not be clearly discriminated (Fig. 6A). All permuted  $R^2Y$  values (goodness of fit: green) and  $Q^2$  values (predictability: blue) on the left were lower than the original point on the right ( $R^2Y$  cum = 0.679,  $Q^2$  cum = 0.401: Fig. 6B) [33]. Although the permutation plot supported the validity of the PLS-DA model, predictability was low [34].

Fig. 7A and B provides the profiles of brain metabolites of the

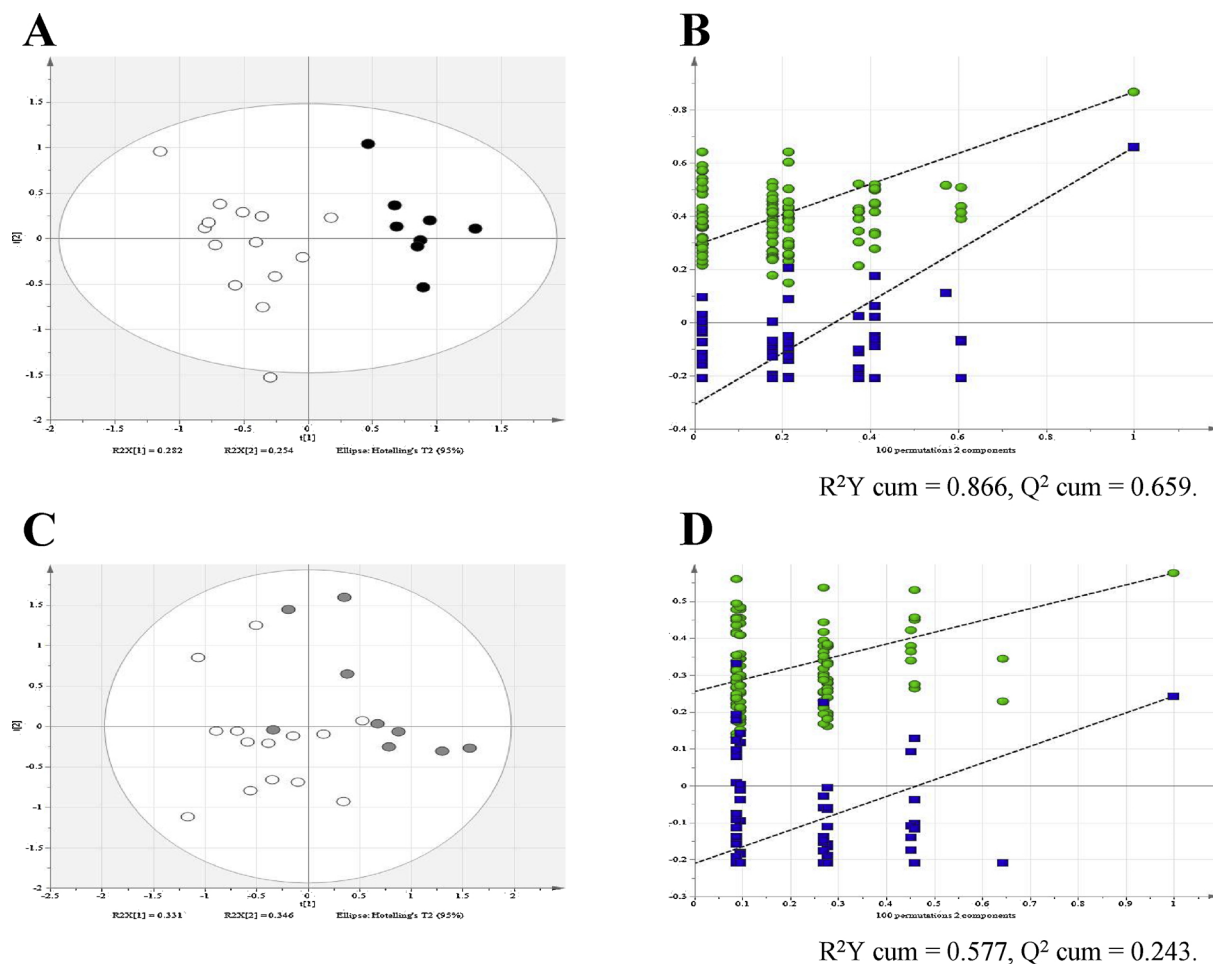
Control Group and the Susceptible Group, as well as the permutation plots. From Fig. 7A, the brain metabolite profiles of the Control Group and the Susceptible Group were clearly distinguishable. Moreover, the  $R^2Y$  cum was 0.866 and the  $Q^2$  cum was 0.659 (Fig. 7B); hence, the fitness and predictability were higher than those in the PLS-DA model of the Control and Stressed Groups. Fig. 7C and D provides the brain metabolite profiles and permutation plots of the Control Group and the Resilient Group. From Fig. 7C, the brain metabolite profiles of the Control Group and the Resilient Group were not clearly distinguishable. From the permutation plots (Fig. 7D), the  $R^2Y$  cum was 0.577, and the  $Q^2$  cum was 0.243; hence, the fitness and predictability had fallen below those of the PLS-DA model of the Control Group and the Stressed Group.

### 3.5. Changes in each brain metabolite in the hippocampus

In this study, we were able to assign 12 kinds of brain metabolites to the NMR spectra. Of these 12, we regarded brain metabolites with *P*-



**Fig. 6.** Influence of stress loading on brain metabolite profiles. (A) Partial least squares discriminant analysis (PLS-DA) score plot: White circles represent the Control Group and black circles represent the Stressed Group. (B) Permutation plot with 100 iterations: Green circles represent fitness ( $R^2Y$  cum = 0.679) and blue boxes represent predictability ( $Q^2$  cum = 0.401).



**Fig. 7.** Changes in brain metabolite profiles in relation to sensitivity to stress. (A) Partial least squares discriminant analysis (PLS-DA) score plot: The white circles represent the Control Group and the black circles represent the Susceptible Group. (B) Corresponding permutation plot with 100 iterations: Green circles represent fitness ( $R^2Y$  cum = 0.866) and blue boxes represent predictability ( $Q^2$  cum = 0.659). (C) PLS-DA score plot: White circles represent the Control Group and the grey circles represent the Resilient Group. (D) Corresponding permutation plot with 100 iterations: The green circles represent fitness ( $R^2Y$  cum = 0.577) and the blue boxes represent predictability ( $Q^2$  cum = 0.243).

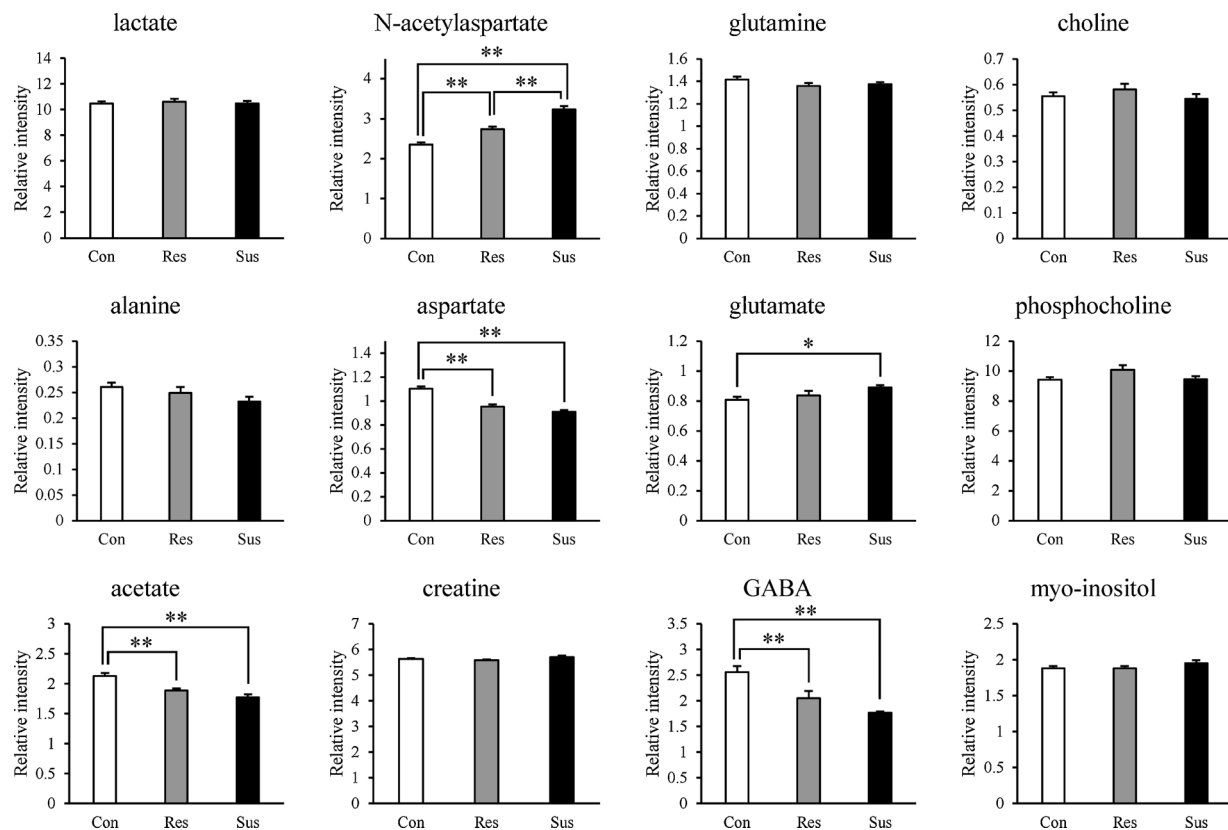
values, as calculated by the Student’s *t*-test, of < 0.05, and lower limits of the 95% confidence interval (CI) of the variable importance in the projection (VIP) score of > 1.0, as ‘potential biomarker candidates [35]’.

**Table 1** (sorted in descending order with respect to VIP score) indicates the relative changes in the brain metabolites in the Stressed Group compared to those of the Control Group. Two metabolites had increased significantly in the Stressed Group, namely N-acetylaspartate

**Table 1**  
Relative changes in each brain metabolite.

Metabolite	Chemical shift (ppm)	Changed <sup>a</sup> (fold difference <sup>b</sup> )	P value	VIP score	VIP score 95%CI
N-acetylaspartate	2.01, 2.49, 2.68	↑ (1.26)	< 0.01	9.01	7.74–10.27
GABA	1.90, 2.29, 3.01	↓ (0.78)	< 0.01	7.64	5.09–10.18
Phosphocholine	3.21, 3.58, 4.16	(1.04)	0.17	5.81	–0.58–12.21
Acetate	1.91	↓ (0.86)	< 0.01	4.40	2.53–6.27
Aspartate	2.68, 2.80, 3.89	↓ (0.85)	< 0.01	2.50	1.69–3.31
Lactate	1.32, 4.10	(1.01)	0.72	1.36	–3.70–6.42
Glutamine	2.14, 2.43, 3.77	(0.96)	0.09	0.84	–0.30–1.97
Myo-inositol	3.27, 3.52, 3.61	(1.02)	0.39	0.67	–0.29–1.62
Alanine	1.47, 3.77	(0.93)	0.11	0.36	–0.94–1.66
Glutamate	2.04, 2.12, 2.33	↑ (1.07)	0.04	0.25	–0.04–0.54
Choline	3.19, 3.51, 4.05	(1.02)	0.69	0.22	–0.06–0.49
Creatine	3.03, 3.92	(1.00)	0.99	0.05	–0.65–0.76

Two-tailed student’s *t*-test was performed on the integrated bucket values of the NMR spectrums between the Control Group and the Stressed Group, to examine the relative variation of brain metabolites. Metabolites with *p* < 0.05 and 95% CI lower limit of the VIP score > 1.0 were considered to be potential biomarker candidates. This table is sorted in descending order of VIP score. <sup>a</sup> Arrows represent an increase or decrease in the metabolite level compared to that of the control group. <sup>b</sup> Fold differences were calculated by ratios of the mean of the integral value of each bucket and are presented in parentheses. GABA:  $\gamma$ -aminobutyric acid; VIP: variable importance in projection; CI: confidence interval.



**Fig. 8.** Relative changes in brain metabolites in relation to sensitivity to stress.

One-way ANOVA was performed for each brain metabolite, followed by a multiple comparison using Tukey-Kramer method. All data are presented as mean  $\pm$  SEM. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ . Con: Control Group ( $n = 14$ ); Res: Resilient Group ( $n = 9$ ); Sus: Susceptible Group ( $n = 8$ ); GABA:  $\gamma$ -aminobutyric acid.

[ $t_{(29)} = -6.17$ ,  $P < 0.01$ ] and glutamate [ $t_{(29)} = -2.20$ ,  $P < 0.05$ ]; and three kinds of metabolites had significantly decreased, namely  $\gamma$ -aminobutyric acid [ $t_{(29)} = 4.12$ ,  $P < 0.01$ ; GABA], acetate [ $t_{(29)} = 5.40$ ,  $P < 0.01$ ], and aspartate [ $t_{(29)} = 7.70$ ,  $P < 0.01$ ]. Of these five metabolites, the metabolites that had a 95% CI lower limit of the VIP score  $> 1.0$  were N-acetylaspartate, GABA, acetate, and aspartate. Therefore, in this study, these four metabolites were identified as potential biomarker candidates that could be used to distinguish between the Control Group and the Stressed Group.

Fig. 8 indicates the changes in 12 brain metabolites among the Control, Resilient, and Susceptible Groups. One-way ANOVA on each brain metabolite revealed statistical significance in N-acetylaspartate [ $F_{(2,30)} = 46.80$ ,  $P < 0.01$ ], aspartate [ $F_{(2,30)} = 32.11$ ,  $P < 0.01$ ], GABA [ $F_{(2,30)} = 12.84$ ,  $P < 0.01$ ], glutamate [ $F_{(2,30)} = 3.96$ ,  $P < 0.05$ ], and acetate [ $F_{(2,30)} = 16.63$ ,  $P < 0.01$ ] among the Control Group, the Resilient Group, and the Susceptible Group. Subsequently, a multiple comparison conducted on these five metabolites using Tukey-Kramer method showed that N-acetylaspartate was the only metabolite that not only increased under stress, but also significantly differed in level between the Resilient Group and the Susceptible Group (all pairwise comparisons =  $P < 0.01$ ). No significant changes were seen in aspartate (test statistic = 1.51,  $P = 0.30$ ), GABA (test statistic = 1.55,  $P = 0.28$ ), or acetate (test statistic = 1.59,  $P = 0.27$ ) between the Resilient Group and the Susceptible Group. Glutamate increased significantly in the Susceptible Group compared to that in the Control Group ( $P < 0.05$ ). No significant changes were observed in any of the remaining seven metabolites.

#### 4. Discussion

In this study, we created preclinical depression model animals that exhibited weight loss, anhedonia, reduced locomotor activity, and

increased anxiety as a result of 4-week stress loading. However, not all of the stressed animals created herein presented with anhedonia, and animals presenting with anhedonia (susceptible) and those not presenting anhedonia (resilient) were mixed together. Susceptible rats presenting with anhedonia exhibited significant decreases in locomotor activities such as crossing and rearing, and a significant increase in anxiety. Resilient rats exhibited a significant decrease in crossing, a decreasing trend in rearing, and an increase in anxiety. Thus, these findings suggest that locomotor activity and anxiety are susceptible to stress regardless of whether the rats were resilient or not.

Mature BDNF alters neuronal survival and synaptic plasticity by binding to tropomyosin-related kinase B (TrkB) receptors [21,36,37]. Atrophy of the hippocampus is observed in patients with major depressive disorders (MDD), with reported BDNF involvement [38,39]. However, researchers have suggested that the BDNF in the hippocampus of MDD patients does not always decrease [40], and no consistent data have been obtained. Mouse and rat BDNF gene consist of at least eight 5' noncoding exons (exons 1–8) and one protein coding 3' exon. In each BDNF transcript, one 5' exon is spliced to the protein coding exon [41]. Ieraci et al. observed the hippocampus of mice exhibiting depressive-like behaviour and reported that there were no changes in BDNF and the total expression of BDNF mRNA, and there was a decrease only in the BDNF-7 mRNA level, out of 8 splice variants [42]. Therefore, while there was no decrease in hippocampal BDNF level in CMS model rats created in this study, there could be a decrease in BDNF splice variants. Moreover, it was suggested that the BDNF level differs between the dorsal and ventral regions of the hippocampus depending on whether the rats acquired resilience to the stress [43]. In this study, since the ventral and dorsal regions of the hippocampus were not studied separately, there is a limitation to assessing the impact of CMS on hippocampal BDNF.

When assessing whether or not a compound acts against depressive-



like symptoms using depression model animals, there is a considerable body of literature describing the use of not only the results of body weight and behavioural tests, but also of the use of changes in BDNF in the hippocampus as evaluation indices [25,44,45]. However, it is also reported that BDNF does not always decrease in the hippocampus of depression model rats that have been created by chronic stress [46]. In this study, we created CMS model animals that manifest symptoms that are close to those observed in clinical situations. However, changes in mature BDNF in the hippocampus were not observed in the Stressed Group or the Susceptible Group. That is, rats in the Stressed Group manifested depression-like behaviours despite the normal amount of mature BDNF in their hippocampus. Therefore, since no consistent data have been obtained from the results of this study or the previously published literature on changes in BDNF in the hippocampus of pre-clinical model animals, it is difficult at the present time to evaluate depressive-like states in relation to the changes in BDNF in the hippocampus or to evaluate the anti-depressive effects of a particular compound.

It was suggested that the brain metabolite profiles of CMS model rats (resilient rats and susceptible rats mixed together) differed from those of the control rats. Previously reported studies demonstrated that profiles of stressed rats and control rats could be clearly distinguished, in contrast to the present study findings [47,48]. However, metabolomic analyses on rats excluding resilient rats revealed a clearer difference in the profiles of brain metabolites between the susceptible rats and the control rats. This implies that mixing in resilient rats made it difficult to distinguish profiles of brain metabolites between CMS model rats and control rats. In other words, brain metabolome analysis could reflect the stress susceptibility.

In the hippocampal tissues of depression model animals that were created in this study, N-acetylaspartate and glutamate levels increased, whereas GABA, acetate, and aspartate levels decreased. Of these five metabolites, four, with the exception of glutamate, were identified as potential biomarker candidates. In addition, no significant differences were observed in stress-induced behaviours such as crossing and grooming, or in brain metabolites such as GABA, acetate, and aspartate, between the Resilient Group and the Susceptible Group. This may imply that these three metabolites may be useful biomarkers for anxiety-like behaviours such as a decrease of crossing and an increase of grooming [30,49].

N-acetylaspartate is present in neurons at high concentrations [50], and is formed from aspartate and acetyl-CoA by aspartate N-acetyltransferase [51]. Acetyl CoA is formed from acetate and coenzyme A. N-acetylaspartate is also known to be a neuronal density marker; thus, the increase in N-acetylaspartate levels in the brain tissue reflects elevated neuronal density [52,53]. Of the 12 metabolites, N-acetylaspartate exhibited the greatest change (a 1.26-fold increase compared to that in the control animals) and had the largest VIP score as well. This supported our previously published work [9], revealing reproducibility of an increase of N-acetylaspartate in CMS model rats. However, some studies found that N-acetylaspartate decreased in the brain of depression model animals [54,55], and there is no consistency in the variation of N-acetylaspartate. Khar et al. and Han et al. demonstrated that N-acetylaspartate in the hippocampus of stress-loaded rats started to increase 24 h from the stress-loading event and gradually returned to the original level [56,57]. In this study, since the sucrose preference test was conducted over a 24-hour span, hippocampus dissection was carried out after at least 24 h from the last stress-loading event. Thus, the fact that consistency was not observed in the variation of N-acetylaspartate in the hippocampus of depression model animals may stem from the change in the levels of N-acetylaspartate with time. In this study, aspartate and acetate, which are the substrates of aspartate N-acetyltransferase, decreased significantly, and N-acetylaspartate, which is the product, increased significantly. These findings suggest that, in the hippocampus of animals in depressive-like states, the activity of aspartate N-acetyltransferase may increase, and that the neuronal

density of the hippocampus may increase. Notably, it is reported that, although depression patients may have reduced hippocampal volumes [58], the hippocampal neuronal density increases by approximately 30% [59]. Moreover, the amount of N-acetylaspartate in the hippocampal tissue of resilient animals fell midway between those of control animals and susceptible animals, suggesting that N-acetylaspartate is a potential biomarker that can distinguish the Control, Resilient, and Susceptible Groups.

GABA is formed from glutamate by glutamate decarboxylase (GAD). GABA activates GABA<sub>A</sub> receptors in adult hippocampal progenitor cells. Reportedly, activated GABA<sub>A</sub> receptors promote neurogenesis and neuronal differentiation, by discharging intracellular Cl<sup>-</sup> and increasing the concentration of Ca<sup>2+</sup> [60,61]. Hemanth et al. reported a decrease in GABA in the hippocampus of CMS model rats [62]. Likewise, GABA decreased significantly in the hippocampus of CMS model rats created in this study. Moreover, the GAD substrate glutamate increased in susceptible rats. These findings raise the possibility that, under stressed state, hippocampal GAD activity could decrease [63] or GABAergic neurons could decrease [64], and the rates of neurogenesis and neuronal differentiation could decrease. Compared to the control animals, both the resilient and susceptible animals exhibited a reduction in GABA. However, glutamate, which is a precursor of GABA, increased only in susceptible animals. This suggests that, in a state that manifests anhedonia, the activity of GAD may be more strongly suppressed.

These considerations reveal that in hippocampal tissues in CMS model rats created in this study, the levels of brain metabolites such as N-acetylaspartate and GABA, which are markers of neuronal density, neurogenesis, or neuronal differentiation, show variations. Thus, brain metabolomic analyses can not only distinguish CMS model rats from control rats, but also indicate the stress susceptibility. Furthermore, the findings of this study suggest that, of the 12 metabolites, N-acetylaspartate may be the only metabolite capable of evaluating the three susceptibility states.

## Declaration of Interest

The authors declare no conflict of interest.

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