Perturbations in Amino Acid Metabolism in Reserpine-Treated Zebrafish Brain Detected by ^1H Nuclear Magnetic Resonance-Based Metabolomics

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Abstract

Depression is a complex and disabling psychiatric disorder, which is expected to be a leading cause for disability by 2030. According to World Health Organization, about 350 million people are suffering with mental health disorders around the globe, especially depression. However, the mechanisms involved in stress-induced depression have not been fully elucidated. In this study, a stress-like state was pharmacologically induced in zebrafish using reserpine, a drug widely used to mediate depression in experimental animal models. Zebrafish received single intraperitoneal (i.p.) injections of 20, 40, and 80 mg/kg body weight reserpine doses and were subjected to open-field test at 2, 24, 48, 72, and 96 h after the treatment. Along with observed changes in behavior and measurement of cortisol levels, the fish were further examined for perturbations in their brain metabolites by ^1H nuclear magnetic resonance (NMR)-based metabolomics. We found a significant increase in freezing duration, whereas total distance travelled was decreased 24 h after single intraperitoneal injection of reserpine. Cortisol level was also found to be higher after 48 h of reserpine treatment. The ^1H NMR data showed that the levels of metabolites such as glutamate, glutamine, histamine, valine, leucine and histidine, lactate, L-fucose, betaine and γ -amino butyric acid (GABA), β -hydroxyisovalerate, and glutathione were significantly decreased in the reserpine-treated group. This study provided some insights into the molecular nature of stress that could contribute toward a better understanding of depression disorder.

Keywords: stress, reserpine-induced, zebrafish, ^1H NMR-based metabolomics, brain metabolites, amino acid metabolism

Introduction

THE WORLD HEALTH ORGANIZATION reported that over 350 million people around the world suffer from mental health disorders, specifically depression.¹ Depression is a complex and disabling psychiatric disorder, expected to be the leading cause of disability by 2030. It has serious social and economic consequences in the form of suicide, medicinal costs, and decreased labor productivity. In the year 2010, globally, the total spending for treating mental and neurological disorders was up to 2.5–8.5 trillion USD.² This cost is estimated to be doubled by 2030 if mitigative actions are not taken. People suffering from depression usually show the

symptoms of psychomotor retardation, anhedonia, reduced appetite, weight loss, reduced cognitive performance, hypothalamus-pituitary-adrenal (HPA) axis (an axis consists of stimulating forward and feedback inhibition loops, involving the brain, pituitary and adrenal glands, which manage glucocorticoid production) dysregulation, increased cardiovascular morbidity, increased amygdala responsiveness, abnormal social behaviour, and altered pain perception.³ Stress-related pathways are directly involved in the pathophysiology of depression.⁴ Understanding stress-induced depression is one of the important approaches to obtain a better understanding of the molecular changes in the pathophysiology of depression.⁵

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Reserpine produces two observable pharmacological effects, that is, sedation and a moderate decline of arterial blood pressure.⁶ The sedative effect of reserpine in cats and dogs was observed after 20–30 min of parenteral administrative dose of reserpine, followed by a decline in motor activity. This action is said to be related to depletion of serotonin and norepinephrine.⁶ Meanwhile, a recent study reported that 1 mg/kg body weight (BW) (subcutaneous) of reserpine induced short-term memory impairment. BW is defined as the measurement taken when the subject is able to stand unassisted using a calibrated scale. In the study, a significant decrease in social interaction and object recognition behavior was observed 3 h after reserpine administration.⁷

In general, drug discovery research is highly time and cost-intensive. Since long, researchers have been using rodents as the translational animal models.⁸ High cost, low throughput, time-consuming, and nonreproducibility of results are some of the limitations of using rodent models in depression studies.⁸ A complementary or additional model may be beneficial to support data generated from rodent studies, toward providing a better understanding of the pathophysiology and etiology of depression.

Zebrafish presents a promising new model for drug discovery. Some characteristics in its favor include its high physiological homology with humans (70% of human genes having at least one obvious zebrafish ortholog), genetic tractability, sensitivity to pharmacological agents, and short reproductive cycle.⁹ Using zebrafish as a disease model to screen for stress is still in its infancy.

Previously, neurochemical and behavioral responses of Unpredictable Chronic Stress (UCS) following developmental isolation was investigated by using zebrafish model.¹⁰ The authors found anxiety-like behavior after induction of UCS in zebrafish, while developmental isolation caused depression and reduced serotonin and dopamine level in the zebrafish brain.¹⁰ There are also many reports on pharmacologically induced depression in zebrafish. Kyzar *et al.* reported hypolocomotion in zebrafish immersed in 40 mg/L of reserpine for 20 min (once in a day) for the period of 7 days.¹¹ Saroya *et al.* gave intraperitoneal (i.p.) injection of reserpine to zebrafish and found only 27% of serotonin than nontreated fish.¹² Intraperitoneal injections are widely used to induce disease symptoms in zebrafish, especially for drugs that are not soluble in water,¹² such as reserpine, whose solubility is 73 mg/L at 30°C.¹³

In this study, we administered i.p. injection to zebrafish to induce stress-like symptoms and investigated the effect of reserpine on zebrafish brain metabolome using ¹H nuclear magnetic resonance (NMR)-based metabolomics approach. Along with results of behavioral and cortisol level analyses, herein, we report the molecular alterations observed in the zebrafish brain metabolome as a result of treatment with reserpine, using ¹H NMR-based metabolomics approach.

Methods

Zebrafish and maintenance

Adult wild-type short fin zebrafish (*Danio rerio*) with weights of 0.5 ± 0.1 g (*n* = 150), of mixed sex (50:50 male:female), were purchased from a local supplier (3B Aquatics, Bandar Baru Bangi, Malaysia). The fish were maintained in dechlorinated holding water (pH 7.0 ± 0.5), at 26°C ± 2°C, in a 14-h light/10-h dark cycle. Fish were fed daily, and twice a day,

with Tetramin Tropical fish flakes. After purchase, the zebrafish were separated based on their gender and were kept in a holding aquarium. After 2 days, the fishes were transferred randomly into four different treatment groups with the ratio 5 male:5 female. The fishes were kept in 2.5 L holding aquarium until the day for depression induction on the 8th day. After reserpine injection, the fishes were transferred into a new aquarium, maintaining the same social group of fishes. Then, behavioral recording was conducted after 24 h and the fishes were collected for whole-body cortisol measurement.

For metabolome evaluation, a new batch of adult zebrafish was purchased and the same steps of acclimatization and reserpine injection were conducted. The fishes were randomly divided into two different groups, that is, control and 80 mg/kg (*n* = 20) groups. To minimize imbalances across multiple factors, all experiments were conducted between 8 am to 1 pm, for every experiment day. All fish used were experimentally naive. Blinding was not feasible in this study since the researcher was conducting the experiment alone. However, behavior experiment was performed on ZebraBox (Viewpoint), equipped with an overhead video camera system and video tracking software to automate behavioral testing and provide unbiased data analysis. The experimental setup and procedures have been approved by the Institutional Animal Care Use Committee (IACUC) of Universiti Putra Malaysia on June 10, 2015 (AUP-R013/2015).

Chemical and reagents

Reserpine (Cat. No. 83580, CAS number 50-55-5), Dulbecco's phosphate-buffered saline (PBS; CAS number 7647-14-5; from Sigma-Aldrich), diethyl ether (CAS number 60-29-7), Parameter Cortisol Assay kit KGE008B (R&D Systems). All other reagents used were of analytical grade. Reserpine was suspended in PBS solution and vortexed for homogenization.

Equipments

Plastic tank (2.5 L), Eppendorf tubes, vortex, centrifuge, pH meter, temperature controller, sponge, fish net and video tracking software (Viewpoint).

Induction of stress-like state in zebrafish model

The schematic diagram of study design is shown in Figure 1. A preliminary study to optimize the dose of reserpine to induce stress-like symptoms in zebrafish was carried out according to Saroya *et al.*¹² Fish were acclimatized for 8 days and underwent fasting for 24 h before induction with reserpine. Based on similar reported studies on zebrafish model, a 3/10-mL U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16") × 31G Short Needle (Becton Dickinson and Company) was used for intraperitoneal injection of reserpine.^{14–17} Before injection, the fish was first anesthetized by immersing in a bath of iced water, with a temperature of about 12°C, until operculum movement ceased or until only the gills were moving. This method is considered less distressful to the fish compared to using tricaine methanesulfonate (MS222).¹⁸

Injection was performed on the anesthetized fish by gently inserting the needle into the midline of the abdomen posterior to the pectoral fins.¹⁵ The injected fish was then placed into the appropriate tank. Fish that did not recover within 60 s of the

FIG. 1. Schematic diagram of stress induction by single i.p. injection of reserpine. i.p., intraperitoneal.

retransfer were excluded from the behavioral test. Fish were injected one at a time and holding water was changed on a daily basis throughout the experiment. After the preliminary experiment, reserpine doses of 20, 40, and 80 mg/kg BW were selected for the full experiment. Fish behavioral analysis was performed at 2, 24, 48, 72, and 96 h after the single i.p. injection of reserpine.

Open-field test

Open-field test was conducted, individually, on four groups of fish, comprising the control group and three reserpine-injected groups (20, 40, and 80 mg/kg), to assess their movement and behavior. Each group consisted of 10 fish ($n=10$, 5 male and 5 female) placed in a test tank measuring $20 \times 8 \times 18$ cm (length $\times$ width $\times$ depth). This number of fish was regarded as an adequate and reliable number of biological replicates for the experiment and the ensuing statistical analysis. Furthermore, the sample size was based on previously reported studies.^{19–21}

The behavioral analysis was done according to Cachat *et al.* with minor modifications.²² The fish were video recorded and analyzed over a 6-min period using Zebrafish Software (Viewpoint) and Smart v3.0, Harvard Apparatus behavioral analysis software. The parameters scored were freezing duration (s) and total distance travelled (cm). Fish were considered frozen when there was no movement for at least 2 s, except for gills and eyes. Total distance travelled was analyzed using Smart v3.0 software using the same video recorded for the freezing duration parameter. Total distance travelled was measured along the experiment detecting the movement of the fish in the selected area. Behavioral data analysis was performed using one-way analysis of variance based on distribution of data, F-distribution.

Measurement of cortisol concentration

Cortisol measurement was performed to assess the effect on the endocrine system after exposure to stressful conditions. Six fish ($n=6$; 3 male and 3 female) from each behavioral test groups were randomly selected after behavioral test, individually snap-frozen in liquid nitrogen, and kept at -80°C for cortisol measurement. As in the open-field test, the number of fish was regarded as an adequate number of replicates for the cortisol analysis. Different numbers of animals per group or replicates have also been used in other studies.^{23–26} The method used for the whole body extraction of cortisol was based on Cachat *et al.*²² Frozen zebrafish samples from behavior testing were thawed and allowed to attain room temperature. Each fish was weighed, cryohomogenized, vortex mixed with 2 mL diethyl ether, and centrifuged for 15 min at 13,000 rpm. The procedure was repeated twice for maximum cortisol extraction, the diethyl ether layer was pooled, evaporated to dryness, and submitted for cortisol assay using a commercial kit, following the procedure provided in the package (Supplementary Data).

^1H NMR metabolomics

The scheme for brain tissue collection and extraction is shown in Figure 2. Fish brain was collected following the procedure established by Tripti & Mullins.²⁶ Briefly, the fish was first anesthetized in 0.2% tricaine, and then euthanized by

incubation in ice water for 15 min. The fish was lightly pat dried with a soft towel, and then pinned onto a dissecting mat through the fleshy part of the tail. The head was removed from the body with a razor blade. Soft tissue was removed from the ventral side of the skull with forceps and the eyes removed using small spring scissors. The skull was then opened and bone from the ventral side of the brain was removed. The head was then placed in a dish of PBS, and skin and skull bones were removed from the dorsal side of the brain.

After dissection, the brain tissues were immediately shock-frozen in liquid nitrogen to stop enzymatic reactions. The average weight of fish brain was 2 mg dry mass/fish. Thus, to improve the spectral quality and sensitivity for NMR analysis, and to ensure efficient identification of the brain metabolites, two fish brains were pooled to represent one sample for the NMR measurements. A similar approach had also been practiced by other researchers, for example, pooled two whole mouse brains as one sample for NMR analysis,²⁷ pooled intestines of twenty fishes for each sample for NMR analysis,²⁸ and pooled four zebrafish telencephalon as one sample for transcriptome analysis.²⁹ The pooled brain tissues were homogenized and stored at -80°C before extraction and NMR measurement. For NMR spectroscopy analysis, the freeze-dried brain tissues were first extracted with a solvent system comprising methanol:H₂O:chloroform (2:1:2), precooled by overnight refrigeration. The extraction mixture was vortexed, sonicated at 530 Hz for 20 min, and finally centrifuged for 15 min at 13,000 rpm. The polar and nonpolar layers were separated and carefully pipetted into labeled Eppendorf tubes, lyophilized, and reconstituted in NMR solvents before subjecting to ^1H NMR spectral measurements.

^1H NMR spectroscopic analysis

Phosphate buffer (pH 7.4) was prepared according to Beckonert *et al.*³⁰ with some modifications. The buffer was prepared in 10 mL instead of 1 L. First, 288.5 mg of Na₂HPO₄, 52.5 mg of NaH₂PO₄, 0.01 mM TSP, and 0.03 mM NaN₃ were added into a 10 mL volumetric flask. Two milliliters of D₂O was added and volume made up to 10 mL with distilled water. The mixture was shaken thoroughly, sonicated at 40°C , and interspersed by shaking the flask, until the salts were dissolved. The buffer solution was prepared fresh before NMR measurements. For NMR sample preparation, deuterated methanol (450 μL) and the phosphate buffer (50 μL) were added to the lyophilized tissue extract. The mixture was centrifuged for 10 min and then transferred to 5 mm diameter NMR tubes for ^1H NMR acquisition.

The ^1H NMR spectra of zebrafish brain extracts were recorded using Bruker Biospin 700 MHz spectrometer, at ambient temperature (25°C). The spectra were run using a Carr-Purcell-Meinboom-Gill (CPMG) pulse sequence, spectral width set to 7500 Hz, and acquired with 128 scans and 32K data points. Free induction decay was zero-filled and multiplied by exponential of line broadening of 0.5 Hz. Deuterium oxide was used as internal lock solvent and a total spin relaxation delay ($2n\tau$) of 10 ms was used to suppress broad signals arising from proteins so that signals from smaller molecules can be clearly seen. Two-dimensional NMR spectra were recorded for the control zebrafish brain extract. The J -resolved (JRES) spectra were acquired using

FIG. 2. Procedure for NMR sample preparation. (A) Fish euthanized in ice water bath (<4°C). (B) Brain tissue collected using microdissecting forceps in PBS solution. (C) Brain tissue shock-frozen in liquid nitrogen and homogenized. (D) Samples stored at -80°C. (E) Samples thawed, weight and solvents added (2:1:2 MeOH:D₂O:CHCl₃). (F) Sonicated for 15–20 min. (G) Centrifuged at 13,000rpm for 10–15 min. (H) Polar layer collected. (I) Freeze-dried for 1–2 days. (J) Deuterated solvent added (300 μL CD₃OD + 300 μL KH₂PO₄ buffer in D₂O) and centrifuged for 5 min. (K) Supernatant transferred to NMR tube. (L) NMR measurement (Bruker 700 MHz). NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline.

eight scans, 12,800 μs increments for delay, and over a spectral width of 16.6041 ppm. The heteronuclear single quantum coherence spectra were obtained using 96 scans, 34.28 μs increments, and over a spectral width of 14.9995 and 165.6580 ppm in the proton and carbon dimensions, respectively.

Data preprocessing and multivariate data analysis

All ¹H NMR spectra were manually phased, baseline corrected, and referenced to 0.1% TSP as internal standard at 0.0 ppm using Chenomx NMR Suite 5.1 Professional (Edmonton, Canada). Using the same software, residual water peak (region 4.8–5.1 ppm) was removed and the 0.5–10 ppm region was divided into small bins of 0.04 ppm widths. The binned spectra were converted to ASCII files and imported to SIMCA-P 13.0 software package for multivariate data analysis (Principal Component Analysis [PCA] and partial least squares discriminant analysis) and visualization of results. The NMR dataset was Pareto scaled and Hotelling's T₂ was used to eliminate scores above 95% T₂ critical to improve the model fitness. The

score plot was constructed to visualize the separation between groups, while the loading plot was used to identify metabolites that contributed to the group's separation.

Identification of zebrafish brain tissue baseline metabolites

Ten zebrafish brains were pooled and extracted for NMR spectral measurements, to identify the baseline brain metabolites. The examination of the NMR spectra revealed the presence of 26 main metabolites, as listed in Table 1. For the annotation, ¹H and ¹³C chemical shifts were compared to chemical shift values reported in the literature and by querying open access metabolomics databases such as Human Metabolome Database (HMDB),³¹ aided by Chenomx NMR Suite 7.7 (Chenomx, Inc.). The identification was further confirmed by JRES experiment. The differentiating factors were identified from the loading plot in PCA and *p*-value calculated from the binning results. Metabolites that gave significant differences were identified as the potential biomarkers.

TABLE 1. IDENTIFICATION OF METABOLITES FROM ^1H NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (700 MHz)

Peak	Metabolites	δ_H (Multiplicity)
1	Acetate	1.9015 (s)
2	Alanine	3.765 (q, 8.72 Hz), 1.47 (d, 7.31 Hz)
3	GABA	2.976 (m, 4.84), 1.8861 (qu, 6.99 Hz), 2.325 (t, 7.09)
4	Choline	3.046 (s), 3.979 (m, 3.75 Hz), 3.54 (m, 6.49)
5	Creatine	3.044 (s), 3.868 (s), 6.609 (s)
6	Ethanolamine	3.814 (m, 4.26 Hz), 3.14 (m, 5.19 Hz)
7	Glutamate	3.719 (dd, 6.55 Hz), 2.03 (m, 6.88 Hz), 2.112 (m, 9 Hz), 2.131 (m, 8.18 Hz), 2.162 (m, 12.19 Hz)
8	Glutamine	3.757 (t, 6.58 Hz), 2.092 (m, 6.97 Hz), 2.031 (m, 9.17 Hz), 2.427 (m, 8.715), 2.46 (m, 6.36 Hz), 6.85 (s), 7.5338 (s),
9	β -Hydroxyisovaleric acid	1.26 (s), 2.35 (s)
10	Histamine	2.832 (m, 14.084 Hz), 2.876 (m, 6.48 Hz), 3.09 (t, 6.99 Hz)
11	Aspartate	3.814 (dd, 3.85 Hz), 2.712 (dd, 11.23 Hz), 2.662 (dd, 16.067)
12	Histidine	4.16 (dd, 7.43 Hz), 3.144 (dd, 3.79 Hz), 3.145 (dd, 11.4282)
13	Acetamide	1.9925 (s)
14	Valine	0.944 (d, 6.76 Hz), 1.073 (d, 7.05 Hz), 2.13 (m, 7.38 Hz), 3.598 (d, 4.73 Hz)
14	Valine	0.944 (d, 6.76 Hz), 1.073 (d, 7.05 Hz), 2.13 (m, 7.38 Hz), 3.598 (d, 4.73 Hz)
15	Lactate	1.326 (d, 7.13 Hz), 4.00 (q, 6.95 Hz)
16	Creatinine	3.04 (s), 3.87 (s)
17	ATP	6.1 (d, 6.29 Hz), 8.22 (s), 8.55 (s), 4.19 (m), 4.26 (m), 4.35 (m), 4.48 (m)
18	Taurine	3.086 (t, 6.35 Hz), 3.284 (t, 6.27 Hz)
19	Glutathione	4.00 (q, 6.913), 3.626 (m), 2.831 (dd, 12.19, 4.078), 2.473 (m), 2.108 (m)
20	L-Fucose	5.185 (d, 3.39 Hz), 4.528 (d, 8.62 Hz), 4.00 (q, 7.403 Hz), 3.779 (dd, 3.22 Hz, 8.75 Hz), 3.69 (m), 3.72 (dd, 6.56 Hz, 4.71 Hz), 1.23 (d, 7.52 Hz), 1.16 (d, 7.19 Hz)
21	Leucine	0.908 (t, 7.1 Hz), 1.878 (m), 3.6 (m)
22	Glycine	3.41 (s), 1.06 (d, 7.19 Hz)
23	Betaine	3.246 (s), 3.87 (s)
24	Beta alanine	2.322 (t, 6.72 Hz), 3.08 (t, 6.76 Hz)
25	NAAG	2.033 (m), 2.133 (m), 2.33 (t, 6.96 Hz), 2.67 (dd, 3.55, 15.18 Hz), 2.82 (dd, 4.18, 17.3 Hz)
26	3-Hydroxyisobutyric acid	1.07 (d, 7.43 Hz), 2.65 (m), 3.69 (m)

GABA, γ -amino butyric acid.

Results

Behavioral changes and cortisol levels of reserpine-treated zebrafish

The freezing duration and total distance travelled, two parameters selected for assessment of the behavioral changes in reserpine-treated group, compared to the normal fish (control group), are shown in Figure 3. Two hours after i.p. injection of reserpine, a slight increase was found in freezing duration of zebrafish groups treated with 20, 40, and 80 mg/L reserpine (Fig. 3), compared to the control group. However, the difference was not significant [$F_{(5, 42)}=0.916, p=0.48$], whereas 24 h after treatment, reserpine caused a significant increase in freezing duration, compared to the control group [$F_{(5, 42)}=5.393, p=0.001$], and a similar increase in freezing duration was found after 72 h, for all the tested doses [$F_{(5, 42)}=5.181, p=0.001$] (Fig. 3). Contrary to freezing duration, 2 h after reserpine injection, total distance was slightly decreased in treated groups [$F_{(3, 28)}=1.340, p=0.281$] (Fig. 4), whereas after 24 h, fish exposed to all reserpine concentrations showed a significant decrease in total distance travelled [$F_{(3, 32)}=4.184, p=0.013$]. A similar behavioral pattern was also observed after 72 h [$F_{(3, 28)}=2.581, p=0.043$] (Fig. 4). We also determined the blood cortisol level in zebrafish after 48 h of reserpine treatment. A four parameter logistic (4-PL) curve-fit was plotted as Supplementary Figure S1. We found

that cortisol level in reserpine-treated groups was significantly increased compared to the control group ($p=0.019$) as shown in Figure 5.

Pattern recognition analysis using PCA

The ^1H NMR spectral processed data were subjected to PCA to study the key metabolites regulated differently in the reserpine-treated zebrafish brain compared to normal fish (control). PCA is an unsupervised and unbiased class separation method, making it a powerful and well-accepted tool. The remarkable strength of this method compared to other supervised method is that, it can highlight intragroup variation so that the quality of data could be assessed.

PCA analysis was performed on normal (CO) and reserpine-treated (RES) zebrafish collected 72 h after i.p. injection of PBS and reserpine, respectively. The generated three-component model had satisfactory goodness of fit and predictability as the value of R2X was 0.959, while Q2X was 0.91. The score plot showed a clear separation between reserpine-treated group and control. Reserpine-treated group was clustered on the negative side of PC1, whereas control group was projected on the positive side of PC1 (Fig. 6A). Overall, PC1 was the major contributor and able to explain 84% variation in the data. Column loading plot showed the key variables (represents metabolites) responsible for the separation of reserpine-treated and control group in

FIG. 3. Freezing duration in OFT after 2, 24, 48, 72, and 96 h after single i.p. injection of reserpine (20, 40, 80 mg/kg). Data are presented as mean ± SEM (*n* = 10). Level of significance: **p* < 0.05, ****p* < 0.0001. OFT, open-field test; SEM, standard error of the mean.

FIG. 4. Total distance travelled in OFT after 2, 24, 48, 72, and 96 h after single i.p. injection of reserpine (80 mg/kg). Data are presented as mean ± SEM (*n* = 10). Level of significance: **p* < 0.05, ***p* < 0.001, ****p* < 0.0001.

FIG. 5. Zebrafish endocrine response (whole body cortisol, ng/g fish) to reserpine (80 mg/kg) i.p. injection. Data are presented as mean $\pm$ SEM ($n=6$). *** $p < 0.0001$.

FIG. 6. PCA of the zebrafish brain, control (light color) and reserpine induced (dark color). (A) Score scatter plot. (B) Loading column plot. In loading column plot: 1, valine; 2, valine/L-fucose/glycine; 3, L-fucose; 4, β -hydroxyisovaleric acid; 5, lactate; 6, alanine; 7, GABA; 8, acetamide; 9, glutamate; 10, glutamine; 11, glutamate/valine/glutathione; 12, histamine/glutathione; 13, betaine; 14, glutamate; 15, choline/histamine; 16, lactate/glutathione/L-fucose; 17, ATP. GABA, γ -amino butyric acid; PCA, Principal Component Analysis.

score plot (Fig. 6B). Valine, β -hydroxyisovaleric acid, lactate, alanine, γ -amino butyric acid (GABA), glutamate, glutamine, histamine, betaine, glutathione, choline, and ATP were identified as important metabolites whose regulation was observed to be perturbed in the zebrafish brain, after reserpine injection. All the identified chemical shifts or columns (represents metabolites) in column loading plot present on the positive side of PC1 corresponded to control group clustered on the positive side of PC1 in score plot.

Discussion

Preclinical and clinical studies have shown that chronic stress could lead to the development of depression.³² Recently, an interesting finding was reported on the induction of fibromyalgia in an animal model upon reserpine administration.³³ Fibromyalgia is one of the most common musculoskeletal pain condition that relates to mitochondrial dysfunction and excessive production of reactive oxygen intermediates, leading to nociceptive, fatigue, and depressive-like behavior. In validating animal model for hypothesis testing and to study the pathogenesis of a disease, the most important thing that needs to be considered is that modeled symptoms are expected to have homology with human disease and can respond to clinically effective treatment within clinically relevant time courses. Knowledge of pathological mechanism is very important in this aspect.³⁴

There are some existing models that have already been considered for stress and depression study, such as rodents, primates, and the latest model being used, the zebrafish.⁸ The use of rodents as translational model for stress and depression began since 1978.³⁵ However, using rodents for disease modeling has several limitations, such as high costs, low throughput, and time-consuming.⁹ A complementary model to the rodents would certainly be a beneficial addition toward obtaining a better insight into the physiopathology of stress and depression. The prevailing rationale is that the animal model reflects level of helplessness or despair, anhedonia, and social withdrawal, all of which are relevant to human depression.³⁵ Apart from behavior analysis, an animal study should include exploration at molecular level, including genetic and epigenetic, so that the study will give clearer insights toward finding more effective medicinal therapy for depression.³⁶

The zebrafish is a new promising model in drug discovery research.⁹ This is due to several unique characteristics, including high physiological homology to human (70% of human gene have at least one obvious zebrafish ortholog), high-throughput value, genetic tractability, low cost, sensitive to various drugs and pharmacological agents (such as ethanol), and a short reproductive cycle.³⁷

Previously, neurochemical and behavioral responses of UCS following developmental isolation were investigated by using a zebrafish model.¹⁰ The study found that UCS protocol only increased anxiety-like behavior, while developmental isolation after UCS caused depression-like behavior and reduced serotonin and dopamine levels in zebrafish brain. In this study, we found significant increase in freezing duration and less distance travelled by zebrafish injected with reserpine compared to nontreated (control) fish. The effects of pharmacologically induced depression on locomotor activity of zebrafish have also been reported previously. Kyzar *et al.* reported that immersion in 40 mg/L of reserpine for 20 min

daily caused hypolocomotion in zebrafish after 7 days.¹¹ Meanwhile, Saroya *et al.* reported that intraperitoneal injection of reserpine was also carried out in zebrafish to study the bystander effect of reserpine-treated fish.¹²

Some of the major symptoms of depression are subjective feeling that cannot be modeled using laboratory animal models. Thus, a more practical way to develop an animal model for depression is by using behavioral analysis that is translated from the symptoms of depressions such as motor responses to stress, reward-related response, and social interaction. Our results showed that, compared to immersion, intraperitoneal injection of reserpine showed early hypolocomotion in zebrafish and persists till 96 h of injection.

Besides behavioral analyses, cortisol is a hormone secreted when the corticotropin-releasing hormone (CRH) is disinhibited by suppressing subgenual prefrontal cortex during stress.⁶ In this study, we found significantly higher levels of cortisol in fish treated with reserpine. One of the key roles of the cortisol in stress response is it activates amygdala to promote arousal and anxiety.⁶ Hypoactivity of the hypothalamic CRH neuron usually occurs in a typical depression state. In this mechanism, low levels of cortisol lead to disinhibition of the immune system. Patients with Addison disease potentially have depressive disorder because of this hypocortisolism.²⁶ In this study, the higher level of cortisol in reserpine-treated fish, compared to the control group, indicated that reserpine was able to induce a stress-like state in the fish model. These results were also in good agreement with previous studies, which also showed increased production of cortisol in zebrafish, while exposed to different stressors.^{38–40} Similar to human, stress responses in zebrafish are mediated by cortisol activated by the cascade of hypothalamic-pituitary hormones and acting through glucocorticoid receptors.⁴¹

Previously, clinicians utilized only a small part of the metabolome such as glucose and cholesterol quantification to monitor diabetic and cardiovascular disease in a patient.⁴² However, currently, the repertoire of biochemical (or small molecules) present in cells, tissues, and biofluids can be captured by metabolomics as the indicator of the global state of organism.^{42,43} In clinical studies of investigation on depression biomarkers, different biofluids were used. Some researchers used plasma,^{44,45} while others used urine⁴⁶ and postmortem human brain.⁴⁷ However, the usage of postmortem brain was limited; it is crucial to understand the pathological process involved. Herein, we used ¹H NMR-based metabolomics approach to study the perturbations in brain metabolites of the fish model, to gain a better understanding of the pathophysiology of the stress-like state of the reserpine-treated fish, which could possibly lead to depression.

¹H NMR-based metabolomics is a powerful and versatile approach to ascertain metabolic signature from complex biofluid and tissue samples. This approach can help in understanding the pathophysiological mechanism underlying disease state as biomarker discovery tool. In this study, metabolites that differentiate the reserpine-treated group from the normal fish could be identified from the multivariate data analysis to the ¹H NMR chemical shift data of the fish brain extracts. Table 2 lists the brain metabolites observed to be significantly changed ($p \leq 0.001$) after treatment with reserpine. These included several amino acids (glutamate, glutamine, histamine, valine, histidine, and β -hydroxyisovalerate), energy-related substances (lactate and L-fucose), osmolytes and organic acids (betaine and GABA), and others, such as glutathione.

TABLE 2. LIST OF METABOLITES THAT DISTINGUISHED BETWEEN RESERPINE-TREATED AND CONTROL ZEBRAFISH GROUPS

Metabolites	Control vs. reserpine-treated	
	Log ₂ (FC)	p
Amino acids		
1 Glutamate	0.7038	0.001
2 Glutamine	0.6772	0.001
3 Glycine	0.2513	0.05
4 Histamine	0.7801	0.001
5 Valine	0.749	0.001
6 Histidine	0.7765	0.001
Energy-related substance		
1 Lactate	0.7376	0.001
2 L-fucose	0.7049	0.002
Osmolytes and organic acids		
1 Betaine	0.7246	0.001
2 GABA	0.6581	0.002
Others		
1 Glutathione	0.5155	0.002
2 β -hydroxyisovalerate	0.6993	0.001

Alteration of amino acid level has been detected in the pathophysiology of depression in rodent model⁴⁸ and human.⁴⁹ Our results showed that reserpine treatment altered the chemistry of zebrafish brain, where amino acids featured significantly among the affected metabolites. The metabolites observed to be significantly decreased in reserpine-treated zebrafish include glutamate ($p=0.001$), glutamine ($p=0.001$), histamine ($p=0.001$), valine ($p=0.001$), histidine ($p=0.001$), lactate ($p=0.001$), L-fucose ($p=0.002$), betaine ($p=0.001$), GABA ($p=0.002$), beta-hydroxyisovaleric acid ($p=0.001$), and glutathione ($p=0.002$), as shown in Figure 7.

Histidine and histamine

L-histidine is an essential amino acid that plays a vital role in histamine synthesis.⁵⁰ Histidine and histamine play important roles in immune system.⁵¹ In human, histaminergic system plays a vital role in many physiological functions, for example, homeostasis of energy and endocrine, sleep-wake cycle, motor and sensory function, cognition, and attention.⁵² Studies showed that interaction of histaminergic and serotonergic system is the main system involved in controlling appetite, emotion, cognition, and sleep.⁵³ Alteration in this system can cause various neuropsychiatric disorders such as Alzheimer's disease, Huntington Disease, and depression disorder.⁵⁴ Histamine is also a neurotransmitter, and disruption of histamine neurotransmission affects behavioral response.⁵⁵

In this study, histamine showed the largest fold change (0.7801) among metabolites with a significant t -test ($p=0.001$) between control and stress-like zebrafish brain model. Kano *et al.* reported a significant decrease in histamine H₁ receptor binding in depressed patients and suggested an important role of histaminergic neuro system in pathophysiology of depression.⁵⁴ Similar to results reported in rodent model⁵⁶ and human,⁵³ we also observed a decreased histamine level in zebrafish brain treated with reserpine, compared to the control group.

FIG. 7. Box plot of main metabolites that showed significant changes in reserpine-induced zebrafish brain metabolites compared to control. The data were analyzed using Kruskal-Wallis ANOVA. ANOVA, analysis of variance.

Choline

Choline is produced by acetylcholine degradation in cholinergic synapses.⁵⁷ Deficit in cholinergic function is usually related to memory impairment. Clinical studies showed inconsistent findings in choline level in depressed patient's brain compared to normal brain. A review reported that 10 out of 19 studies (various part of brain) showed significant differences in choline level in depressed patients compared to normal individuals.⁵⁶ Among five studies focused on frontal lobe, three reported significant reduction of choline in depressed patients compared to control, while the other two reported contradictorily. Riley and Renshaw also investigated mesial temporal region of the human brain with respect to choline regulation.⁵⁶ They found higher level of choline in mesial temporal region of depressed patients. Seven out of 19 of these studies showed no significant difference of choline level in depressed patients compared to control in pre-frontal lobe and cortex, including the dorsolateral prefrontal cortex, anterior cingulate cortex, hippocampus, and occipital cortex. A study conducted by Diego-Adeliño *et al.* showed 13% higher choline level in chronic depression than in the first episode depressive patient.⁵⁸ However, our results indicated no significant difference in choline concentrations between reserpine-treated and control zebrafish brain.

Glutamine/glutamate

Glutamate plays an important role as major excitatory neurotransmitter in the central nervous system, which involved in

memory encoding and storage.⁵⁹ Besides that, it has numerous other important roles in brain, such as metabolic precursor for the major inhibitory neurotransmitter (GABA).⁶⁰ Another important role of glutamatergic system is to regulate HPA axis, and further regulating stress response.⁶¹ Abnormal glutamatergic neurotransmission was believed being the pathophysiology of depression.⁶² In normal brain, glutamate is synthesized from glutamine by glutaminase.⁵⁹ A 3-year-follow-up clinical study reported low glutamine and glutamate levels in depressed patient than normal individuals.⁶² In another study, glutamate was reported to be significantly reduced in pre-frontal cortex of stressed mice.⁶¹ There are also some other brain disorders caused by glutamatergic abnormalities such as schizophrenia and bipolar disorder.⁶³ In this study, we also found significantly lower level of glutamate in reserpine-treated zebrafish compared to the control group ($p=0.001$). So our findings are in line with previous clinical studies regarding the regulation of glutamate in depression.

Lactate

The disturbance in energy metabolism was also found to be an inducer of depression in rat model of depression.⁶⁴ Glucose is the main source of energy in brain, and is crucial for synthesis of neurotransmitters and other functions of the brain cells. Detka *et al.* found higher lactate level in prenatal stressed rat (a model of depression).⁶⁵ A similar pattern was also found in human cerebral spinal fluid,

which showed higher ventricular lactate in depressed adolescents compared to control.⁶⁶ However, we found lower level of lactate in reserpine-treated group compared to the control group. Chen *et al.* used multiplatform metabolomics approach (NMR and gas chromatography-mass spectrometry) to differentiate severity of depression in human, and identified 14 urine metabolites responsible for distinguishing moderate depression from control and 22 metabolites for distinguishing patients having severe depression than normal people.⁶⁷ The study reported down-regulation of N-methylnicotinamide, acetone, choline, malonate, and glycerophosphocholine in moderately depressed patients than normal people, while fructose, nicotinate, citrate, isobutyrate, ribose, vanillic acid, sorbitol, and azelaic acid were found to be upregulated.⁶⁸ In severe depression, α -ketoglutarate, trimethylamine-N-oxide (TMAO), indoxyl sulfate, m-hydroxyphenyl acetate, malonate, 3-hydroxyphenylacetic acid, N-methylnicotinamide, and oxaloacetate were decreased, while nicotinate, *p*-hydroxyphenyl acetate, sucrose, alanine, taurine, choline, citrate, hydroxylamine, myristic acid, formate, isobutyrate, palmitic acid, glycine, and lactate were found higher in severely depressed patients. From this result, lactate was found higher in patients with severe depression, but not in moderately depressed patients. Meanwhile, Zheng and coworkers reported higher levels of some energy-related metabolites, for example, α -ketoglutarate and succinate, while lactate, glucose, acetate, and pyruvate were found to be decreased in urine of patients in the first episode of depression.⁴⁶ In this study, a single i.p. injection of reserpine on zebrafish also caused similar reduction in lactate level in the stress-induced zebrafish brain.

β -Hydroxyisovaleric acid

β -hydroxyisovaleric acid is a volatile fatty acid. A clinical study reported that isovaleric acid has correlations with human depression.⁶⁸ This metabolite is usually found in the gut as a result of bacterial fermentation.⁶⁸ It was also reported that isovaleric acid can directly access the brain, and can inhibit Na^+ , K^+ , and ATPase, which are crucial enzymes in maintaining normal neurotransmission. Furthermore, β -isovaleric acid was also shown to be significantly increased in children having chronic psychosocial stress and gut problem.⁶⁹ In this study, most of the differentiating brain metabolites between reserpine-treated zebrafish compared to the control group are related to amino acid metabolism. However, other metabolites such as β -hydroxyisovaleric acid and lactate were also found to be important metabolites affected by reserpine treatment, indicating their involvement in the regulation of the stress-like state in the fish model. Recently, isovaleric acid has also been reported to be involved in regulation of depression in rodents and human.⁶⁸

Conclusion

This study demonstrated the induction of a stress-like state in adult zebrafish. Behavioral and ^1H NMR metabolomics data showed that single i.p. injection of reserpine can successfully induced stress-like behaviour in zebrafish. Reserpine i.p. injection significantly increased the freezing duration, but the effect was eliminated after 72 h. Total distance travelled

was significantly decreased after 80 mg/kg i.p. injection of reserpine. Amino acids, osmolytes, organic acids, and several energy-related metabolites were implicated in the stress-like state of the zebrafish, resulting from reserpine treatment. This study represents our initial attempt at obtaining a better understanding of the molecular changes in the pathophysiology of the stress-depression connection, based on zebrafish model. To the best of our knowledge, this is the first time that ^1H NMR-based metabolomics was used to investigate the altered brain chemistry of reserpine-treated zebrafish. Although the NMR technique was successful in detecting a modest number of altered metabolites, which could be potential biomarkers for the disorder, a much more robust and sensitive analytical platform, notably liquid chromatography mass spectrometry (LCMS/MS)ⁿ and other similar techniques, may be needed for detection of other brain metabolites such as the neurotransmitters serotonin, dopamine, and norepinephrine. Nevertheless, the biochemical information obtained in this study was in good agreement with previous reports on biomarkers of stress-induced depression identified in rodents and human. Meanwhile, the employability of zebrafish to model depression looks good and should be explored further, particularly for its potential use in screening for antidepressant drugs.

Authors' Contributions

K.S., M.T.A., W.N.W.I., and I.S.I. designed the experiment; F.Z. performed all the experiments; N.A.B. and A.M. assisted in zebrafish and NMR experiments, respectively; M.T.A., M.M., and S.S. provided expertise in statistical analysis; W.N.W.I., I.S.I., and H.A. provided intellectual input; and K.S., F.Z., and M.T.A. wrote the article.

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Supplementary Material

Supplementary Data
Supplementary Figure S1

References

1. Akimoto H, Oshima S, Ohara K, Negishi A, Hiroshima H, Nemoto T, *et al.* Metabolic profiling of hippocampal tissue in rats with depression-like symptoms. *Biol Pharm Bull* 2018;40:789–796.
2. Chisholm D, Sweeny K, Sheehan P, Rasmussen B, Smit F, Cuijpers P, *et al.* Scaling-up treatment of depression and anxiety: a global. *Lancet Psychiatry* 2016;3:415–424.
3. Neumann ID, Wegener G, Homberg JR, Cohen H, Slattery DA, Zohar J, *et al.* Animal models of depression and anxiety:

- what do they tell us about human condition? *Prog Neuropsychopharmacol Biol Psychiatry* 2011;35:1357–1375.
4. Gold PW. The organization of the stress system and its dysregulation in depressive illness. *Mol Psychiatry* 2014; 20:1–16.
 5. van Praag HM. Can stress cause depression? *World J Biol Psychiatry* 2005;6(Suppl 2): 5–22.
 6. Milton HA, Werner F, Otto K. Pharmacology of reserpine and its implications for anesthesia. *Anesthesiology* 1963; 24:524–542.
 7. Pereira AG, Poli A, Matheus FC, LB da Silva, Fadanni GP, Izídio GS, *et al.* Temporal development of neurochemical and cognitive impairments following reserpine administration in rats. *Behav Brain Res* 2020;383:1–7.
 8. Nguyen M, Stewart AM, Kalueff AV. Aquatic blues: modeling depression and antidepressant action in zebrafish. *Prog Neuropsychopharmacol Biol Psychiatry* 2014;55: 26–39.
 9. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, *et al.* The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 2013;496: 498–503.
 10. Fulcher N, Tran S, Shams S, Chatterjee D, Gerlai R. Neurochemical and behavioral responses to unpredictable chronic mild stress following developmental isolation: the zebrafish as a model for major depression. *Zebrafish* 2017; 14:23–24.
 11. Kyzar E, Stewart AM, Landsman S, Collins C, Gebhardt M, Robinson K, *et al.* Behavioral effects of bidirectional modulators of brain monoamines, reserpine and d-amphetamine in zebrafish. *Brain Res* 2013;1527:108–116.
 12. Saroya R, Smith R, Seymour C, Mothersill C. Injection of reserpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: confirmation in vivo of a role for serotonin in the mechanism. *Dose Response* 2010;8:317–330.
 13. Yalkowsky SH: *Aqueous Solubility Data*. CRC Press, Taylor and Francis Group, Boca Raton, FL, 2010.
 14. Neely MN, Pfeifer JD, Caparon M. Streptococcus-zebrafish model of bacterial pathogenesis. *Infect Immun* 2002;70: 3904–3914.
 15. Phelps HA, Runft DL, Neely MN. Adult zebrafish model of streptococcal infection. *Curr Protoc Microbiol* 2009;13: Chapter 9, Unit 9D.1.
 16. Richetti SK, Blank M, Capiotti KM, Piatto AL, Bogo MR, Vianna MR, *et al.* Quercetin and rutin prevent scopolamine-induced memory impairment in zebrafish. *Behav Brain Res* 2011;217:10–15.
 17. Borvinskaya E, Gurkov A, Shchapova E, Baduev B, Meglinski I, Timofeyev M. Distribution of PEG-coated hollow polyelectrolyte microcapsules after introduction into the circulatory system and muscles of zebrafish. *Biol Open* 2018;7:1–12.
 18. Wilson JM, Bunte RM, Carty AJ. Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). *J Am Assoc Lab Anim Sci* 2009;48:785–789.
 19. Wong RY, Oxendine SE, Godwin J. Behavioral and neurogenomic transcriptome changes in wild-derived zebrafish with fluoxetine treatment. *BMC Genomics* 2013;14:1–13.
 20. Yoon C, Yoon D, Cho J, Kim S, Lee H, Choi H, *et al.* ¹H NMR-based metabolomics studies of bisphenol A in zebrafish (*Danio rerio*). *J Environ Sci Health B* 2017;52:282–289.
 21. Pittman J, Hylton A. Behavioral, endocrine, and neuronal alterations in zebrafish (*Danio rerio*) following sub-chronic coadministration of fluoxetine and ketamine. *Biochem Behav Part B* 2015;139:158–162.
 22. Cachat J, Stewart A, Grossman L, Gaikwad S, Kadri F, Chung KM, *et al.* Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nat Protoc* 2010;5:1786–1799.
 23. Rambo CL, Mocelin R, Marcon M, Villanova D, Koakoski G, de Abreu MS, *et al.* Gender differences in aggression and cortisol levels in zebrafish subjected to unpredictable chronic stress. *Physiol Behav* 2017;171:50–54.
 24. Reolon GK, de Melo GM, da Rosa JGS, Barcellos LJG, Bonan CD. Sex and the housing: effects on behavior, cortisol levels and weight in zebrafish. *Behav Brain Res* 2018; 336:85–92.
 25. de Abreu MS, Giacomini ACVV, Genario R, dos Santos BE, Marcon L, Demin KA, *et al.* The impact of housing environment color on zebrafish anxiety-like behavioral and physiological (cortisol) responses. *General and Comparative Endocrinology* 2020;294:113499.
 26. Gupta T, Mullins MC. Dissection of organs from the adult zebrafish. *J Vis Exp* 2010;37:1–5.
 27. Liu J, Ann Sheldon R, Segal MR, Kelly MJS, Pelton JG, Ferriero DM, *et al.* ¹H nuclear magnetic resonance brain metabolomics in neonatal mice after hypoxia-ischemia distinguished normothermic recovery from mild hypothermia recoveries. *Pediatr Res* 2013;74:170–179.
 28. Qiao R, Sheng C, Lu Y, Zhang Y, Ren H, Lemos B. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. *Sci Total Environ* 2019;662:246–253.
 29. Huang V, Butler AA, Lubin FD. Telencephalon transcriptome analysis of chronically stressed adult zebrafish. *Sci Rep* 2019;9:1–9.
 30. Beckonert O, Keun HC, Ebbels TMD, Bundy J, Holmes E, Lindon JC, *et al.* Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc* 2007;2:2692–2703.
 31. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, *et al.* HMDB: the Human Metabolome Database. *Nucleic Acids Res* 2007;35:521–526.
 32. Belleau EL, Treadway MT, Pizzagalli DA. The impact of stress and major depressive disorder on hippocampal and median prefrontal cortex morphology. *Biol Psychiatry* 2019;85:443–453.
 33. da Silva Brum E, Fialho MFP, Fischer SPM, Hartmann DD, Gonçalves DF, Scussel R, *et al.* Relevance of mitochondrial dysfunction in the reserpine-induced experimental fibromyalgia model. *Mol Neurobiol* 2020;57:4202–4217.
 34. Kalueff AV, Tuohimaa P. Experimental modeling of anxiety and depression. *Acta Neurobiol Exp* 2004;64:439–448.
 35. Duman CH. Models of depression. *Vitam Horm* 2010;82: 1–21.
 36. Abelaira HM, Réus GZ, Quevedo J. Animal models as tools to study the pathophysiology of depression. *Braz J Psychiatry* 2013;35:112–120.
 37. Stewart AM, Cachat J, Gaikwad S, Robinson KS, Gebhardt M, Kalueff AV. Perspectives on experimental models of serotonin syndrome in zebrafish. *Neurochem Int* 2013;62: 893–902.
 38. Alderman SL, Bernier NJ. Ontogeny of the corticotropin-releasing factor system in zebrafish. *Gen Comp Endocrinol* 2009;164:610–669.

39. Alsop D, Vijayan MM. Development of the corticosteroid stress axis and receptor expression in zebrafish. *Am J Physiol Regul Integr Comp Physiol* 2008;294:711–719.
40. Ramsaya JM, Feista GW, Vargab ZM, Westerfieldb M, Kentd ML, Schreck CB. Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture* 2009;297:157–162.
41. Pavlidis M, Theodoridi A, Tsalafouta A. Neuroendocrine regulation of the stress response in adult zebrafish, *Danio rerio*. *Prog Neuropsychopharmacol Biol Psychiatry* 2015; 60:121–131.
42. Quinones MP, Kaddurah-Daouk R. Metabolomics tools for identifying biomarkers for neuropsychiatric diseases. *Neurobiol Dis* 2009;35:165–176.
43. Johnson CH, Ivanisevic J, Siuzdak G. Metabolomics: beyond biomarkers and towards mechanisms. *Nat Rev Mol Cell Biol* 2016;17:451–459.
44. Shi B, Tian J, Xiang H, Guo X, Zhang, L. A ¹H-NMR plasma metabonomic study of acute and chronic stress models of depression in rats. *Behav Brain Res* 2013;241:86–91.
45. Liu, X, Zheng P, Zhao X, Zhang Y, Hu C, Li J, *et al.* Discovery and validation of plasma biomarkers for major depressive disorder classification based on liquid chromatography-mass spectrometry. *J Proteome Res* 2015; 14:2322–2330.
46. Zheng, P, Wang Y, Chen L, Yang D, Meng H, Zhou D, *et al.* Identification and validation of urinary metabolite biomarkers for major depressive disorder. *Mol Cell Proteomics* 2013;12:207–214.
47. Zhang R. Metabolomic profiling of post-mortem brain reveals changes in amino acid and glucose metabolism in mental illness compared with controls. *Comput Struct Biotechnol J* 2016;14:106–116.
48. Zhou X, Liu L, Zhang Y, Pu J, Yang L, Zhou C, *et al.* Metabolomics identifies perturbations in amino acid metabolism in the prefrontal cortex of the learned helplessness rat model of depression. *Neuroscience* 2016;343:1–9.
49. Woo HI, Chun MR, Yang JS, Lim SW, Kim MJ, Kim SW, *et al.* Plasma amino acid profiling in major depressive disorder treated with selective serotonin reuptake inhibitors. *CNS Neurosci Ther* 2015;21:417–424.
50. Yoshikawa T, Nakamura T, Shibakusa T, Sugita M, Naganuma F, Iida T, *et al.* Insufficient intake of L-histidine reduces brain histamine and causes anxiety-like behaviors in male mice. *J Nutr* 2014;144:1637–1641.
51. Best J. A mathematical model for histamine synthesis, release, and control in varicosities. *Theor Biol Med Model* 2017;14:1–19.
52. Shan L, Bao A, Swaab DF. The human histaminergic system in neuropsychiatric disorders. *Trends Neurosci* 2015;38:167–177.
53. Munari L. Brain histamine is crucial for selective serotonin reuptake inhibitors' behavioral and neurochemical effects. *Int J Neuropsychopharmacol* 2015;18:1–10.
54. Kano M, Fukudo S, Tashiro A, Utsumi A, Tamura D, Itoh M, *et al.* Decreased histamine H1 receptor binding in the brain of depressed patients. *Eur J Neurosci* 2004;20:803–810.
55. Du H, Zhang H, Zhao Y, Liu M, Chen A, Liu S, *et al.* Metabolic analysis of the antidepressive effects of Yangxinshi Tablet in a vascular depression model in mice. *Biomed Chromatogr* 2017;32:1–9.
56. Riley CA, Renshaw PF. Brain choline in major depression: a review of the literature. *Psychiatry Res Neuroimaging* 2018;271:142–153.
57. Albiñana E, Luengo JG, Baraibar AM, Muñoz MD, Gandía L. Choline induces opposite changes in pyramidal neuron excitability and synaptic transmission through a nicotinic receptor-independent process in hippocampal slices. *Pflügers Arch* 2017;469:779–795.
58. Diego-Adelino J, de Portella MJ, Gómez-Ansón B, López-Moruelo O, Serra-Blasco M, Vives Y, *et al.* Hippocampal abnormalities of glutamate/glutamine, N-acetylaspartate and choline in patients with depression are related to past illness burden. *Biol Psychiatry Cogn Neurosci Neuroimaging* 2013;38:107–116.
59. Bruno D, Nierenberg J, Cooper TB, Marmar CR, Zetterberg H, Blennow K, *et al.* Neurobiology of learning and memory: the recency ratio is associated with reduced CSF glutamate in late-life depression. *Neurobiol Learn Mem* 2017;141:14–18.
60. Niciu MJ, Ionescu DF, Richards EM, Zarate C. Glutamate and its receptors in the pathophysiology and treatment of major depressive disorder. *J Neural Transm (Vienna)* 2013; 121:907–924.
61. Wang W, Guo H, Zhang SX, Li J, Cheng K, Bai SJ, *et al.* Targeted metabolomic pathway analysis and validation revealed glutamatergic disorder in the prefrontal cortex among chronic social defeat stress mice model of depression. *J Proteome Res* 2016;15:3784–3792.
62. Hashimoto K, Bruno D, Nierenberg J, Marmar CR, Zetterberg H, Blennow K, *et al.* Abnormality in glutamine–glutamate cycle in the cerebrospinal fluid of cognitively intact elderly individuals with major depressive disorder: a 3-year follow-up study. *Transl Psychiatry* 2016;6:744–746.
63. Ramadan S, Lin A. Glutamate and glutamine: a review of in vivo MRS in the human brain. *NMR Biomed* 2014;26:1–36.
64. Chen G, Yang D, Yang Y, Li J, Cheng K, Tang G, *et al.* Amino acid metabolic dysfunction revealed in the prefrontal cortex of a rat model of depression. *Behav Brain Res* 2015;278:286–292.
65. Detka J, Kurek A, Kucharczyk M, Głombik K, Basta-Kaim A, Kubera M, *et al.* Brain glucose metabolism in an animal model of depression. *Neuroscience* 2015;295:198–208.
66. Radley KA, Mao X, Case JA, Kang G, Shungu DC, Gabbay V. Increased ventricular cerebrospinal fluid lactate in depressed adolescents. *Eur Psychiatry* 2016;32:1–8.
67. Chen J, Zhou CJ, Zheng P, Cheng K, Wang HY, Li J, *et al.* Differential urinary metabolites related with the severity of major depressive disorder. *Behav Brain Res* 2017;332:280–287.
68. Szczesniak O, Hestad KA, Hanssen JF, Rudi K. Isovaleric acid in stool correlates with human depression. *Nutr Neurosci* 2016;19:279–283.
69. Michels N, Wiele VD, Henauf S. Chronic psychosocial stress and gut health in children: associations with calprotectin and fecal short-chain fatty acids. *Psychosom Med* 2017;9:927–935.

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