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The International Journal on the Biology of Stress

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ists20

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To cite this article: Aikaterini Iliou, Angeliki-Maria Vlaikou, Markus Nussbaumer, Dimitra Benaki, Emmanuel Mikros, Evangelos Gikas & Michaela D. Filiou (2021) Exploring the metabolomic profile of cerebellum after exposure to acute stress, Stress, 24:6, 952-964, DOI: 10.1080/10253890.2021.1973997

To link to this article: https://doi.org/10.1080/10253890.2021.1973997

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ORIGINAL RESEARCH REPORT

Exploring the metabolomic profile of cerebellum after exposure to acute stress

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ABSTRACT

Psychological stress and stress-related disorders constitute a major health problem in modern societies. Although the brain circuits involved in emotional processing are intensively studied, little is known about the implication of cerebellum in stress responses whereas the molecular changes induced by stress exposure in cerebellum remain largely unexplored. Here, we investigated the effects of acute stress exposure on mouse cerebellum. We used a forced swim test (FST) paradigm as an acute stressor. We then analyzed the cerebellar metabolomic profiles of stressed ($n = 11$) versus control ($n = 11$) male CD1 mice by a Nuclear Magnetic Resonance (NMR)-based, untargeted metabolomics approach. Our results showed altered levels of 19 out of the 47 annotated metabolites, which are implicated in neurotransmission and N-acetylaspartic acid (NAA) turnover, as well as in energy and purine/pyrimidine metabolism. We also correlated individual metabolite levels with FST behavioral parameters, and reported associations between FST readouts and levels of 4 metabolites. This work indicates an altered metabolomic signature after acute stress in the cerebellum and highlights a previously unexplored involvement of cerebellum in stress responses.

ARTICLE HISTORY
Received 15 April 2021
Accepted 24 August 2021

KEYWORDS
FST; cerebellum; metabolomics; NMR; acute stress; mouse

Introduction

Stress is a risk factor for all chronic conditions (Chrousos, 2009) and stress-related disorders are the leading cause of disability worldwide (World Health Organization, 2021). Brain circuits involved in emotional and stress stimuli processing mainly include communication among cortical regions, the hippocampus and amygdala nuclei (Price & Drevets, 2010). The cerebellum is a brain region that predominantly controls movement and has been to date rarely discussed in the context of emotional processing. Emerging evidence suggests that the cerebellum is involved in stress regulation due to its connection with stress-relevant circuits and the ability to process stress-related neurochemical signals (Moreno-Rius, 2019). In particular, the cerebellum is a neuron-rich brain region (Herculano-Houzel, 2009) which contributes to several non-motor functions (Moreno-Rius, 2018, 2019, 2019) and has well-documented interactions with key brain regions involved in stress responses, including hippocampus (Rochefort et al., 2011), amygdala (Farley et al., 2016) and hypothalamus (Zhu et al., 2006). Acute stress has been recently reported to affect sensorimotor adaptation driven by cerebellum in humans (Gheorghe et al., 2018). Early studies in rodents investigating stress-induced brain changes have also indicated that acute stressors alter serotonin levels (Curzon & Green, 1971; Takada et al., 1995) and increase c-fos mRNA expression in rat cerebellum (Bozas et al., 1997). Furthermore, cold swim stress resulted in increased circular guanosine monophosphate levels in mouse cerebellum (Dinnendahl, 1975), whereas rats showed decreased cerebellar benzodiazepine receptor density upon swim stress (Bitran et al., 1998) and changes in cerebellar muscarinic cholinergic receptor binding ability upon acute forced swimming (Estevez et al., 1984).

Here, we investigated the effects of exposure to acute stress in the mouse cerebellum metabolome. As an acute stressor, we used the forced swim test (FST), which is a well-established stress paradigm of an uncomfortable, inescapable situation where mice are placed in a beaker with water and swim for a determined amount of time (Can et al., 2012; de Kloet & Molendijk, 2016). We explored the molecular underpinnings of exposure to this stressor in the cerebellum using metabolomics. Metabolomics assesses low molecular weight molecules in biological fluids in a high-throughput manner and is one of the recent advances in the field of "omics," measuring the end products of cell activity and thus reflecting the effects of both intrinsic and environmental factors (Nicholson & Lindon, 2008). Central nervous system

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Supplemental data for this article can be accessed here.

metabolomics has recently emerged as an important tool for understanding the underlying mechanisms of neuropsychiatric and stress-related disorders (Quinones & Kaddurah-Daouk, 2009). Unique metabolic profiles have been observed in different brain regions, reflecting their distinct susceptibility to stress exposure (Choi et al., 2018), however, little is known on the effect of stress on the cerebellum metabolome. In this study, we implemented a Nuclear Magnetic Resonance (NMR)-based untargeted metabolomics approach to analyze metabolomic profile changes following acute stress in cerebellum, a largely unexplored area in the context of stress responses.

Methods

Animals

Experiments were carried out with male CD1 mice, group housed in 4–6 animals per group, in the animal facility of the University of Ioannina, Greece. All animals were 7 weeks old and housed under standard conditions (12 h light/12 h dark cycle, lights on at 7 a.m., temperature maintained at 23 ± 2 °C and humidity at 55 ± 5%) with standard bedding and nesting material, in polycarbonate cages (42 × 26 × 15 cm). Water and food were provided *ad libitum*. Mouse experiments were approved by the local authorities and all procedures were carried out in accordance with the European Communities Council Directives 2010/63/EU. Mice were divided into two groups: stressed mice that were exposed to an acute stress paradigm (FST) ($n = 11$) and unstressed controls ($n = 11$), that were not exposed to acute stress.

Acute stress paradigm

At 7 weeks of age, mice belonging to the stress group were subjected to FST, which was used as an acute stressor. Each mouse was placed into a 2 L glass beaker filled with tap water, 22 ± 1 °C, up to the height of 1.6 L. Mice were unable to touch the bottom of the beaker or escape for a 6 min testing period, during which the following parameters were recorded: time of struggling, swimming, floating and latency to the 1st floating event. FST behavioral scoring was performed by the same experienced experimenter to minimize inter-individual bias. During recovery, mice were returned to their home cages containing bedding with *ad libitum* access to food and water. Acute stress experiments were performed between 8 a.m. and 1 p.m.

Cerebellum sampling

Stressed mice were euthanized 120 min after FST initiation, allowing a recovery period in accordance with established protocols (Floriou-Servou et al., 2018; Jiang et al., 2004; Picard et al., 2015) to detect persistent metabolite changes upon exposure to acute stress. All mice were anesthetized, the cerebellum was dissected, tissue was snap frozen in liquid nitrogen and stored at −80 °C.

Metabolite extraction

Deuterium oxide (D_2O) was purchased from Deutero GmbH (Kastellaun, Germany). Trimethylsilyl Propionate (TSP) was used as internal standard in NMR analysis and was purchased from Sigma-Aldrich-Merck (Darmstadt, Germany). Chloroform and methanol were purchased from Sigma-Aldrich-Merck. All chemicals were of analytical grade and dissolved in ultrapure water (ddH_2O). Cerebella were allowed to thaw on ice and were homogenized in an ice-cold chloroform/methanol solution [2:1 (*v/v*), 1 mL 100 mg tissue] using an Ultra-Turrax homogenizer (IKA-Werke GmbH & Co., Staufen im Breisgau, Germany) with a S 10 N-5 G dispersing tool suitable for small volumes at 20,000 rpm (increasing gradually) for 3 min at room temperature followed by sonication for 5 min on ice. Equal volume of ice-cold ultrapure water was added and the samples were vortexed for 1 min, left on ice for 15 min and centrifuged at 18,900 g for 20 min at 4 °C. The two phases were collected, and the extraction procedure was repeated for the pellet. A 1.4 mL aliquot of the polar phase was dried in a vacuum centrifuge for 9 h. The dried extracts were reconstituted in 650 μL of a mixture consisting of 10% phosphate buffer, pH 7.4, prepared in D_2O (containing TSP as internal standard and sodium azide as preservative) and 90% D_2O and vortexed for 1 min. Of each sample, 550 μL were aliquoted and transferred in a 5 mm diameter NMR tube.

NMR metabolomic analysis

NMR experiments were performed employing a Bruker AVANCE III 600 MHz NMR Spectrometer (BrukerBioSpin GmbH, Karlsruhe, Germany). 1 D ^{1}H NMR experiments were acquired using the noesy-pre-saturation pulse sequence, with gradients (noesygppr1d, Bruker library) offering the optimum water suppression. For each ^{1}H 1 D spectrum, 128 scans were acquired with a spectral width of SW = 12335.526 Hz and a sampling of 64k points, resulting in an acquisition time of 2.7 sec. A mixing time of 10 msec was used. Fully automated sample loading, temperature stability, field homogeneity, pulse calibration, data acquisition and processing (including Fourier transform, phase/baseline correction and axis calibration referenced to the chemical shift of TSP at $\delta = 0.00$ ppm) were employed using the IconNMR v. 5.0.7 software (Bruker BioSpin GmbH, Rheinstetten, Germany). TopSpin 3.5 (Bruker BioSpin GmbH, Rheinstetten, Germany) was used for spectra visualization.

Metabolomic data pre-processing

Bucketing of NMR spectra was conducted using MATLAB R2019B (MathWorks, Massachusetts, US). A spectral bucketing of 0.001 ppm was applied at the range of $\delta = 0.90$–9.00 ppm. The water region was removed from the analysis. The remaining spectral points were normalized to total intensity.

Metabolite/pathway identification

The Chenomx NMR suite v.8.4. (Chenomx Inc., Alberta, Canada) was used for metabolite identification. 2 D NMR experiments (multiplicity-edited Heteronuclear Single Quantum Correlation-HSQC-DEPT and Total Correlation Spectroscopy (TOCSY)) in selected samples aided molecular identification. Statistical Total Correlation Spectroscopy (STOCSY), a mathematical technique for the identification of correlated spectral features, was also used to provide latent information for metabolite identification. Pathway enrichment analysis was performed by Over Representation Analysis (ORA). ORA was implemented in MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/) (Chong et al., 2018) using the hypergeometric test and the pathway-associated metabolite sets from The Small Molecule Pathway Database (https://smpdb.ca).

Statistical analysis

Principal Component Analysis (PCA), Partial Least Square-Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) were applied to the binned, normalized NMR data using SIMCA-P 14.0 (Umetrics, Umea, Sweden). The quality of obtained models was assessed by R2X (variance explained by X Matrix) and Q2 (goodness of prediction) obtained via 7x cross-validation, permutation testing was performed using 100 random permutations (PLS-DA and OPLS-DA) and data were pareto-scaled. Univariate analyses were performed using Graph-Pad Prism 7.0 (GraphPad Software, California, US). To assess whether the variables are normally distributed, D'Agostino & Pearson, Shapiro–Wilk and Kolmogorov–Smirnov normality tests were employed. For variables that did not pass at least one of the normality tests, the non-parametric Mann–Whitney test was applied. For peak intensities following normal distribution, the Student's *t*-test was performed (with Welch's correction for unequal variances when necessary, depending on the *F*-test results). False Discovery Rate (FDR) correction was applied for multiple hypothesis testing and the FDR adjusted *p*-values (P_{adj}) were calculated. Spearman's correlation analysis for metabolite intensities and FST behavioral parameters was performed only for the stressed mice using MATLAB R2019b and FDR correction was applied for each behavioral parameter.

Results

Altered metabolite signatures in the cerebellum upon exposure to acute stress

To study the effects of acute stress in mouse cerebellum, we performed a comparative NMR metabolic profiling of stressed versus control mice. A schematic representation of the experimental workflow is shown in Figure 1. We used FST as a stressor. All recorded FST parameters for stressed mice including struggling, swimming, floating and latency to the 1st floating event are provided in detail in Supplementary Table S1.

In total, 47 known metabolites were annotated in the analyzed mouse cerebella. These metabolites are involved in several biological processes, including amino acid metabolism, purine/pyrimidine metabolism, energy and lipid metabolism, as well as neurotransmission. In Figure 2, the 1 D ^{1}H NMR spectra of the two groups under comparison with spectral annotation are shown. Detailed chemical shifts of all annotated metabolites and respective multiplicity of peaks are provided in Supplementary Table S2. 2 D NMR spectra that aided molecular identification are shown in Supplementary Figures S1–S3, while information on the observed cross-peaks is provided in Supplementary Table S3. The STOCSY plot of

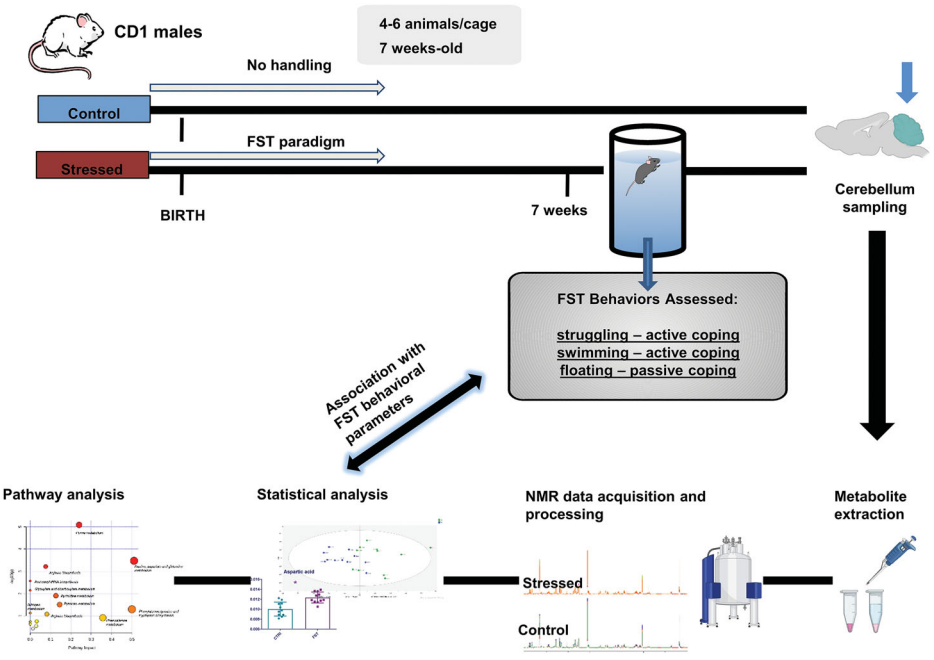

Figure 1. Schematic representation of the experimental workflow. CD1 mice are subjected to FST acute stress ($n = 11$). Unstressed mice are used as controls ($n = 11$). Cerebellum is collected from all mice for untargeted NMR metabolomics analysis. Altered pathways between the stressed and control groups are identified and selected metabolites are correlated with FST behavioral parameters.

Table 1. VIP scores with a value 5 as extracted from the PLS-DA model of the stressed (FST) versus control (CTR) mice, using the untargeted ^{1}H 1D NMR data.

δ (ppm)	Metabolites	VIP
1.919	Acetic acid; 4-aminobutyric acid; arginine	12.59
1.325	Lactic acid; threonine	12.41
1.336	Lactic acid; threonine	12.08
2.022	N-acetylaspartic acid	11.77
1.918	Acetic acid; 4-aminobutyric acid; arginine	10.33
2.023	N-acetylaspartic acid	10.29
3.040	Creatinine; ethanolamine	9.56
3.042	Creatinine; ethanolamine	8.99
1.920	Acetic acid; 4-aminobutyric acid; arginine	8.91
3.207	Choline phosphate	7.57
2.021	N-acetylaspartic acid	7.46
1.326	Lactic acid; threonine	7.30
3.206	Choline phosphate	6.99
3.931	Creatinine	6.88
3.041	Creatinine; ethanolamine	6.40
3.232	sn-Glycerophosphocholine	6.21
1.335	Lactic acid; threonine	6.20
1.337	Lactic acid; threonine	6.04
1.917	Acetic acid; 4-aminobutyric acid; arginine	5.87
2.236	Acetone	5.83
1.921	Acetic acid; 4-aminobutyric acid; arginine	5.78
3.929	Creatinine	5.70
2.024	N-acetylaspartic acid	5.64
4.116	Lactic acid	5.32
3.930	Creatinine	5.26
3.233	sn-Glycerophosphocholine	5.21
3.205	Choline phosphate	5.19
1.327	Lactic acid; threonine	5.14
4.104	Lactic acid	5.00

To each spectral region extracted from the PLS-DA model the annotated metabolite is shown. More than one metabolite may be annotated per bin (ppm), due to peak overlap of the particular spectral region. In this case, annotations are given in the order of signal intensity.

N-acetylaspartic acid (NAA) is shown in Supplementary Figure S4.

Of the 47 annotated metabolites, 19 exhibited significant alterations between stressed and control mice as shown by univariate analysis employing the least overlapped peak, using the binned, normalized NMR data. These include metabolites related to neurotransmission, energy, purine/pyrimidine and amino acid metabolism (Figure 3). Regarding purine/pyrimidine metabolism, stressed mice exhibit lower levels of adenosine ($P_{adj} = 0.01$), adenosine diphosphate (ADP) ($P_{adj} = 0.01$), AMP ($P_{adj} < 0.001$), IMP ($P_{adj} = 0.02$) and uridine monophosphate (UMP) ($P_{adj} = 0.02$), whereas inosine ($P_{adj} = 0.01$), hypoxanthine ($P_{adj} = 0.005$) and uridine diphosphates (UDPs) ($P_{adj} = 0.03$) levels were higher compared to control mice. Neurotransmission alterations in the cerebellum of stressed mice were evidenced by increased levels of gamma-aminobutyric acid (GABA) ($P_{adj} = 0.002$) and ethanolamine ($P_{adj} = 0.003$) as well as decreased levels of NAA ($P_{adj} = 0.02$). The ratio of N-acetylaspartyl-glutamic acid (NAAG) to N-acetylaspartic acid was also significantly increased ($P_{adj} = 0.004$). Amino acids showing statistically significant altered levels after stress exposure included arginine ($P_{adj} = 0.03$), aspartic acid ($P_{adj} = 0.001$), phenylalanine ($P_{adj} = 0.02$) (increased in stressed versus control mice) and glutamine ($P_{adj} = 0.008$) (decreased in stressed versus control mice), all of which are involved in neurotransmission, either as neurotransmitter precursors or as neurotransmitters. Acetic acid ($P_{adj} = 0.001$) and lactic acid ($P_{adj} = 0.01$) levels were increased in stressed mice, whereas acetone ($P_{adj} < 0.001$) and formic acid ($P_{adj} = 0.04$) levels were decreased. Detailed statistics of the metabolomic data are provided in Supplementary Table S4, while the boxplots of the non-significant metabolites are shown in Supplementary Figure S5.

The metabolomic profiles of stressed versus control mouse cerebella are significantly different as this is shown by a multivariate PCA analysis indicating a clear discrimination between the two groups (Figure 4). The PLS-DA analysis confirmed the clear separation of the examined groups (Figure 5(A)), with appropriate quality parameters and prediction power [R2Y (cum) = 0.915 and Q2 (cum) = 0.555], while permutation testing ($n = 100$ permutations) (Figure 5(B)) and ANOVACV-ANOVA ($P_{adj} = 0.006$) proved the validity of the constructed model. Metabolites with Variable Importance in Projection (VIP) greater than 2 were considered as important for the differentiation of the two groups (Supplementary Table S5). Among the metabolites with the highest discrimination power (VIP 5, Table 1) were acetic acid, lactic acid, NAA, creatinine, acetone, choline phosphate and sn-Glycerophosphocholine, with the five first also having been found significant in the univariate analysis. The Loadings Plot (Figure 5(C)), shows spectral bins attributed to acetic acid and lactic acid being elevated in the stressed mice compared to controls, while spectral bins of NAA, creatinine and acetone were decreased in the stressed mice compared to controls. Other metabolites with VIP >2 include amino acids (aspartic acid, glycine, glutamine, threonine, alanine, glutamic acid) as well as metabolites implicated in purine/pyrimidine metabolism (AMP, IMP, inosine, hypoxanthine, uridine). Similar results were obtained from the OPLS-DA analysis, which also accounts for the orthogonal (uncorrelated) variation in the metabolomics data (Supplementary Figure S6). Clear discrimination of the two groups is observed in the cross-validated scores plot of the model, while the S-plot shows the variables with the highest contribution (covariance) and confidence (correlation). Detailed statistics of the PLS-DA and OPLS-DA models are shown in Supplementary Table S6.

Altered molecular pathways in the cerebellum upon exposure to acute stress

Pathway enrichment analysis (Figure 6) of the 19 significant metabolites revealed aspartate metabolism as the most important pathway with $n = 6$ hits and FDR corrected $P_{adj} = 0.002$, followed by urea cycle ($n = 5$, $P_{adj} = 0.006$), purine metabolism ($n = 7$, $P_{adj} = 0.006$), glutamate metabolism ($n = 5$, $P_{adj} = 0.04$), ammonia recycling ($n = 4$, $P_{adj} = 0.04$) and amino sugar metabolism ($n = 4$, $P_{adj} = 0.04$) (Supplementary Table S7).

Acute stress-induced metabolite alterations correlate with behavioral readouts

We also examined the associations of cerebellum metabolite levels with the FST behavioral parameters measured for the stressed mice. Annotated metabolite levels were correlated with the four FST recorded behavioral parameters; struggling, swimming, floating and latency to the 1st floating (Figure 7). Four of the examined metabolites; NAA, threonine, tyrosine and UMP, exhibit strong association (FDR level at f = 5%)

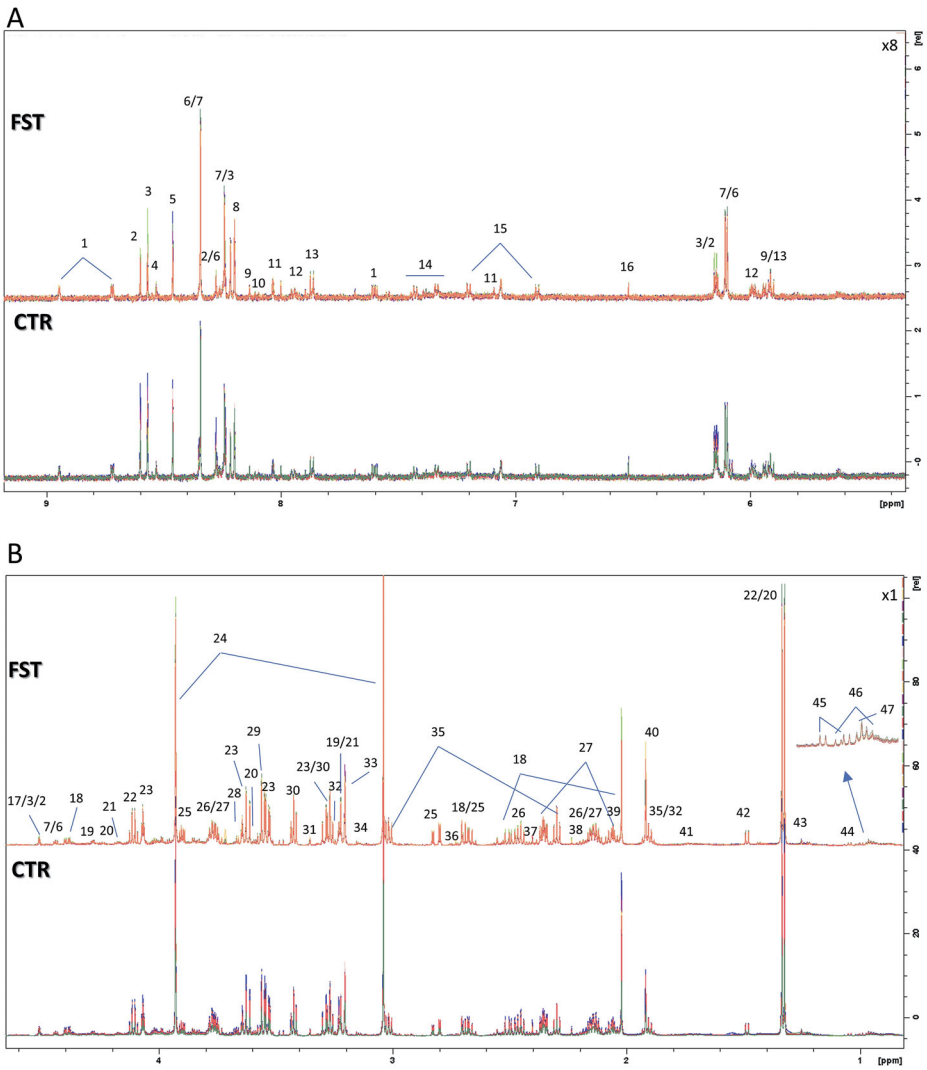

Figure 2. 1D ^{1}H NMR spectra of stressed and control groups. (A) The aromatic region (scaled x8), (B) The aliphatic region. (1) Nicotinuric acid, (2) AMP, (3) IMP, (4) ADP, (5) Formic acid, (6) Adenosine, (7) Inosine, (8) Hypoxanthine, (9) Guanosine Triphosphate (GTP), (10) UMP, (11) Histidine, (12) UDPs, (13) Uridine, (14) Phenylalanine, (15) Tyrosine, (16) Fumaric acid, (17) Ascorbic acid, (18) NAA, (19) sn-Glycerophosphocholine, (20) Threonine, (21) Choline phosphate, (22) Lactic acid, (23) Myo-inositol, (24) Creatinine, (25) Aspartic acid, (26) Glutamine, (27) Glutamic acid, (28) Glycerol, (29) Glycine, (30) Taurine, (31) Methanol, (32) Arginine, (33) Choline, (34) Ethanolamine, (35) GABA, (36) Malic acid, (37) Succinic acid, (38) Acetone, (39) NAAG, (40) Acetic acid, (41) Lysine, (42) Alanine, (43) 3-Hydroxyisovaleric, (44) Propylene glycol, (45) Valine, (46) Isoleucine, (47) Leucine.

with behavioral parameters, while a weaker association (FDR level at f = 10%) is also observed for acetic acid, ADP, aspartic acid, glutamine, hypoxanthine, IMP, leucine, methanol and uridine. Among the four examined FST parameters, swimming and floating are those with the strongest association with the aforementioned metabolites of stressed mice. More specifically, NAA and UMP were associated positively with swimming ($P_{adj\ NAA} = 0.04$, $r_{NAA} = 0.81$; $P_{adj\ UMP} = 0.04$, $r_{UMP} = 0.79$) and negatively with floating ($P_{adj\ NAA} = 0.01$, $r_{NAA} = -0.86$; $P_{adj\ UMP} = 0.03$, $r_{UMP} = -0.82$), while the opposite effect was observed for threonine (swimming: $P_{adj} = 0.04$, $r = -0.83$, floating: $P_{adj} = 0.001$, $r = 0.91$) and tyrosine (swimming: $P_{adj} = 0.045$, $r = -0.76$, floating: $P_{adj} = 0.07$, $r = 0.72$).

Discussion

In the present study, we investigated the metabolic signature upon acute stress exposure in the mouse cerebellum. We used FST as an acute stressor and we analyzed changes in the cerebellum metabolome by NMR. We included a recovery time between the stressor and sample collection in order to detect persistent metabolite changes in the cerebellum, also taking into consideration the organism's adaptive responses to stress. Acute stress effects persist at the molecular level for hours, as evidenced by studies investigating -omic profile alterations after acute stress exposure (Floriou-Servou et al., 2018; Jiang et al., 2004).

FST is an established, strong acute stressor in rodents (de Kloet & Molendijk, 2016). In addition to being a reliable predictor of antidepressant activity, FST exerts well-characterized changes in stress markers such as plasma/serum corticosterone levels (Drossopoulou et al., 2004). A single FST exposure following a 2 h recovery phase, as used in our study, is able to induce neurochemical and metabolomic changes in the brain (Jiang et al., 2004). Effects of FST acute stress exposure have been also shown to be strain- and sex-dependent (Bielajew et al., 2003). Recent findings indicate an

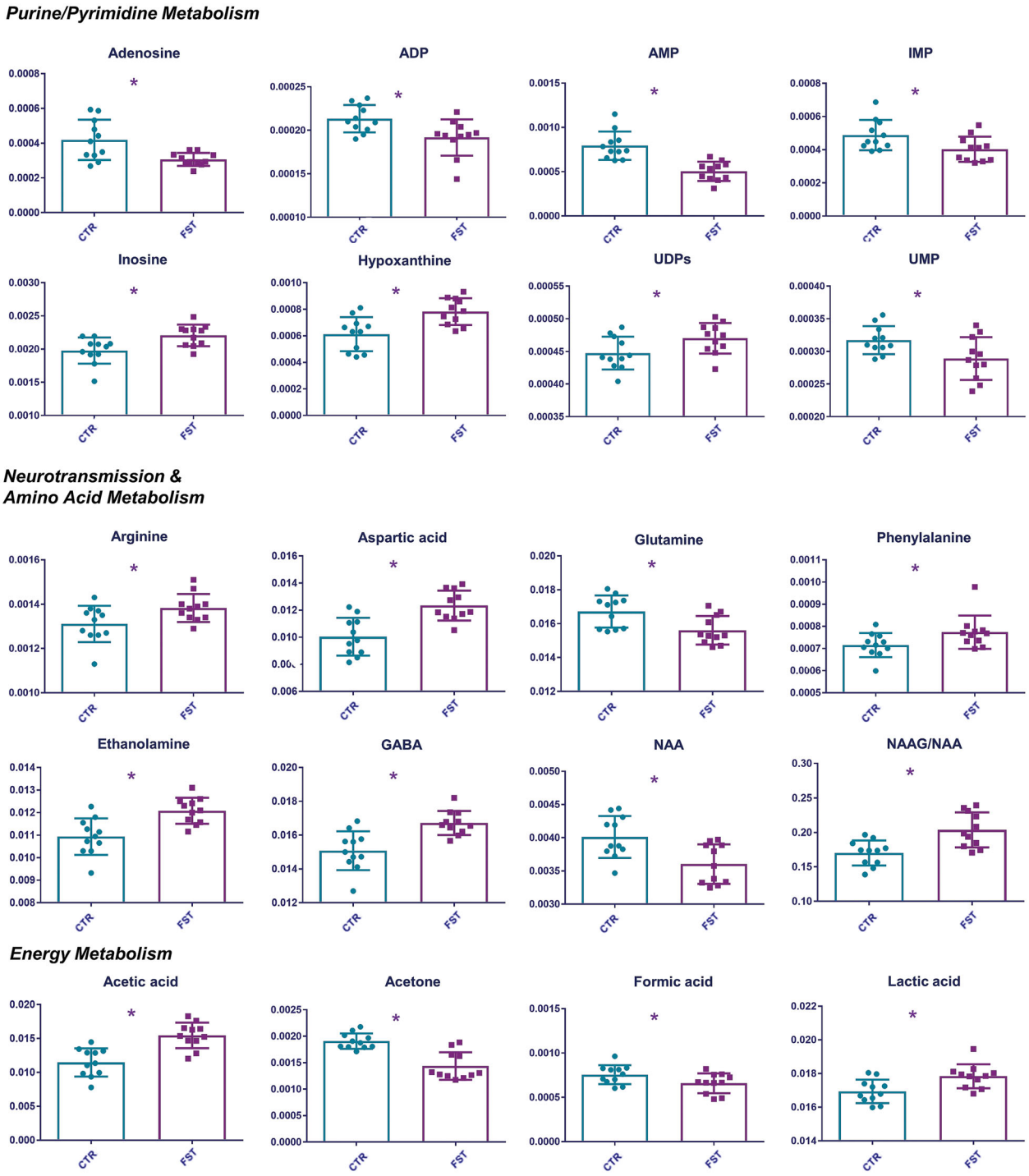

Figure 3. Boxplots of the statistically significant metabolite level alterations between stressed (FST) and control (CTR) mice classified according to pathways; purine/pyrimidine metabolism, neurotransmission and amino acid metabolism, and energy metabolism. NAA: N-acetylaspartic acid; NAAG: N-acetylaspartyl-glutamic acid.

increased activity of dopaminergic neurons in the prefrontal cortex and hippocampus of male rats exposed to FST compared to females. This hyperactivity of neurons may reflect an adaptive mechanism in response to stress (Dalla et al., 2008).

Energy demands for each brain region are associated with functional processes and the power needed for action potentials. In addition, the energy use required for different subcellular processes is determined by the neural information that is differentially computed in the cerebral and cerebellar areas of the brain (Howarth et al., 2012). Although the predominant function of the cerebellum is motor-related (Ghez & Fahn, 1985), functional imaging studies support that cerebellar activation is related to language, attention and mental imagery (Stoodley & Schmahmann, 2009). In addition, correlation studies have shown interactions between the cerebellum and non-motor cerebral cortex areas (Buckner et al., 2011).

Changes in brain areas are mediated by a series of neurochemical messengers inducing behavioral effects. The

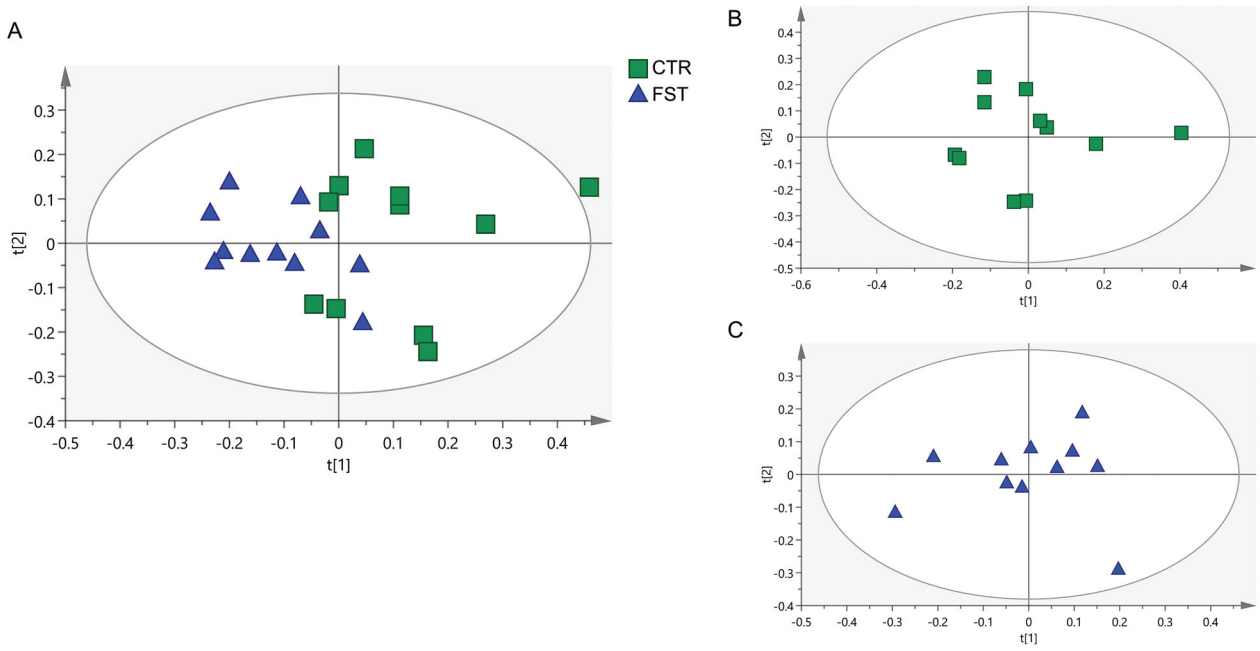

Figure 4. (A) PCA Scores Plot of stressed (FST) versus control (CTR) mice. A clear discrimination of the examined groups is observed, (B) PCA-Class Scores Plot for the control (CTR) group, (C) PCA-Class Scores Plot for stressed (FST) group. In PCA-Class analysis, no outliers are observed in each group. (▲): Stressed (FST), (■): Control (CTR).

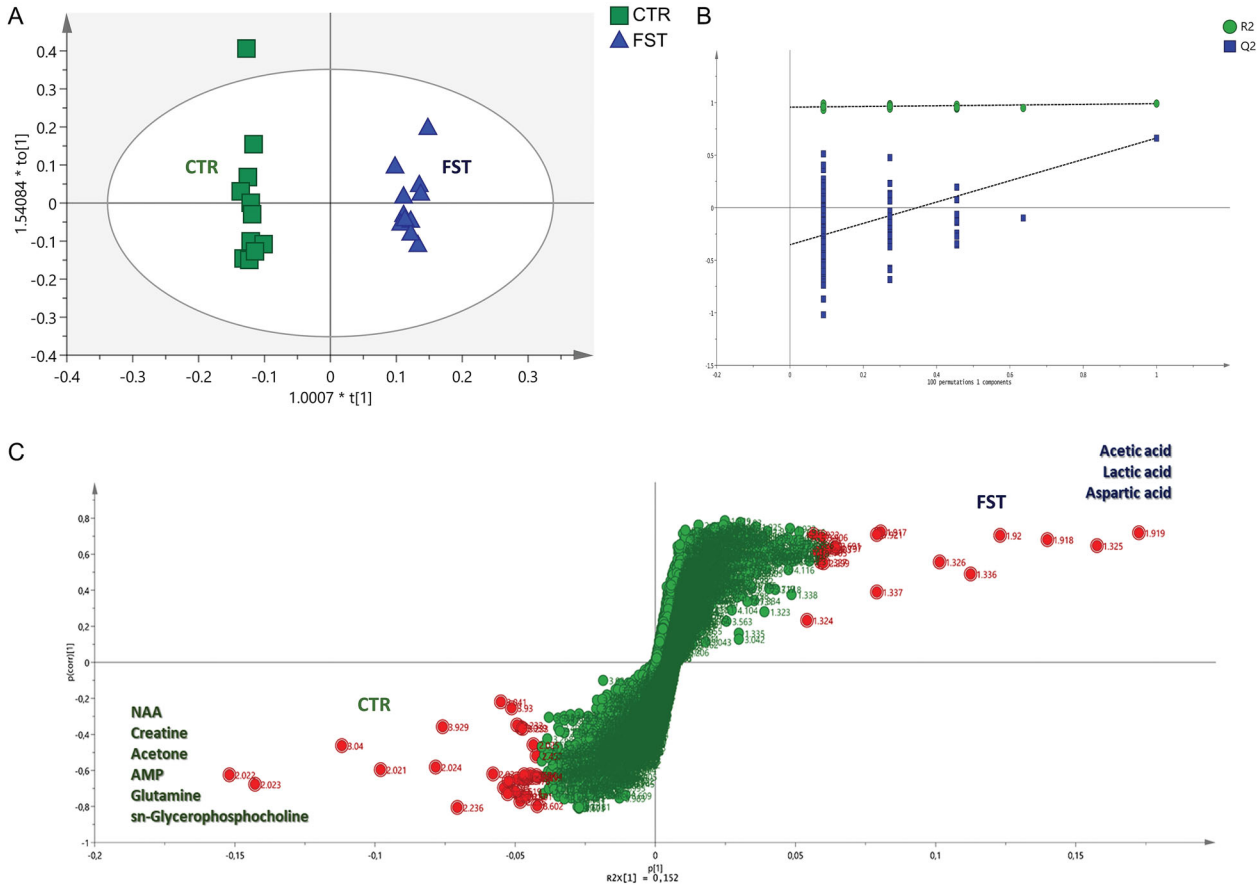

Figure 5. PLS-DA analysis of stressed (FST) versus control (CTR) mice. (A) The cross-validated Scores Plot shows clear discrimination of groups, (B) Permutation test ($n = 100$ permutations) supports the validity of the constructed model, (C) Loadings Plot of the PLS-DA analysis. Loadings on the right side are increased in the stressed group and belong mostly to lactic acid and acetic acid variables (shown as ppm). Loadings on the left side are increased in the control group and belong mostly to NAA, creatinine and acetone variables. (▲): Stressed (FST), (■) Control (CTR); NAA: N-acetylaspartic acid.

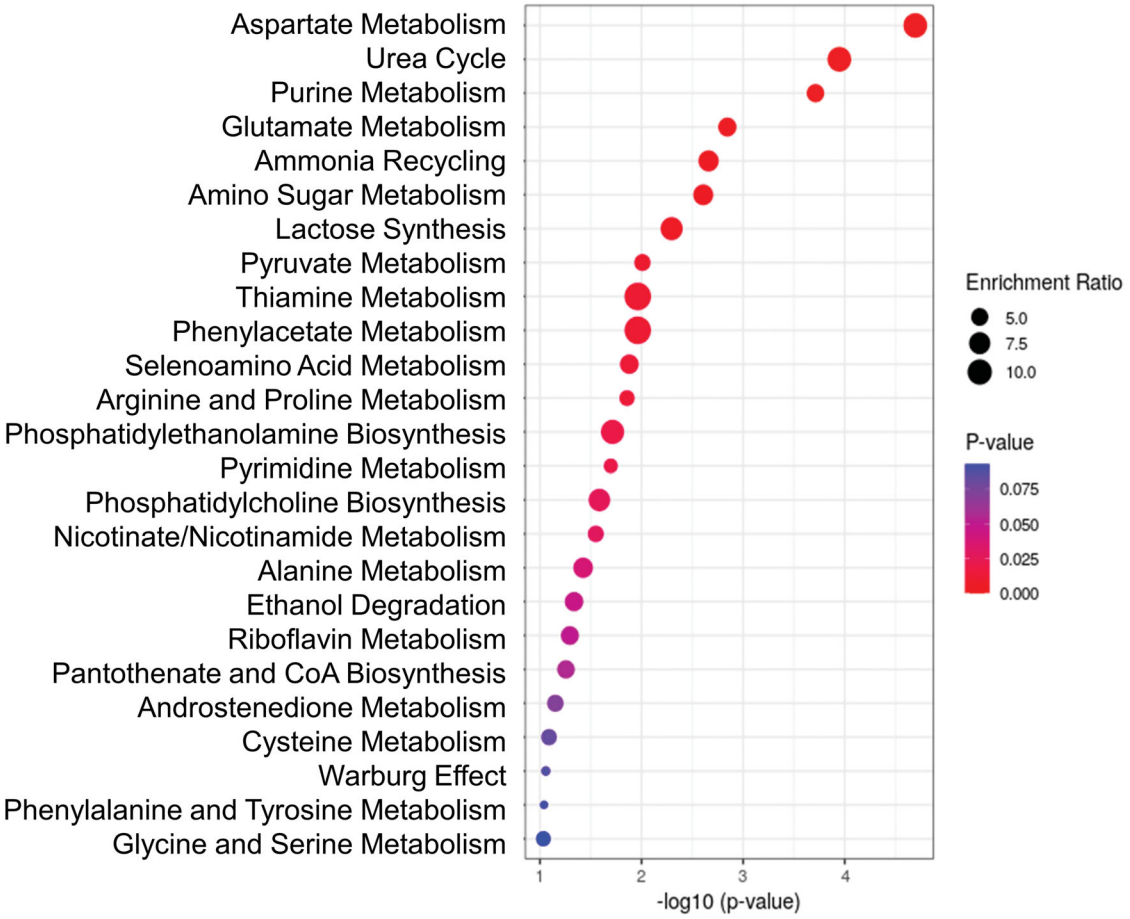

Figure 6. Summary plot for pathway enrichment analysis.

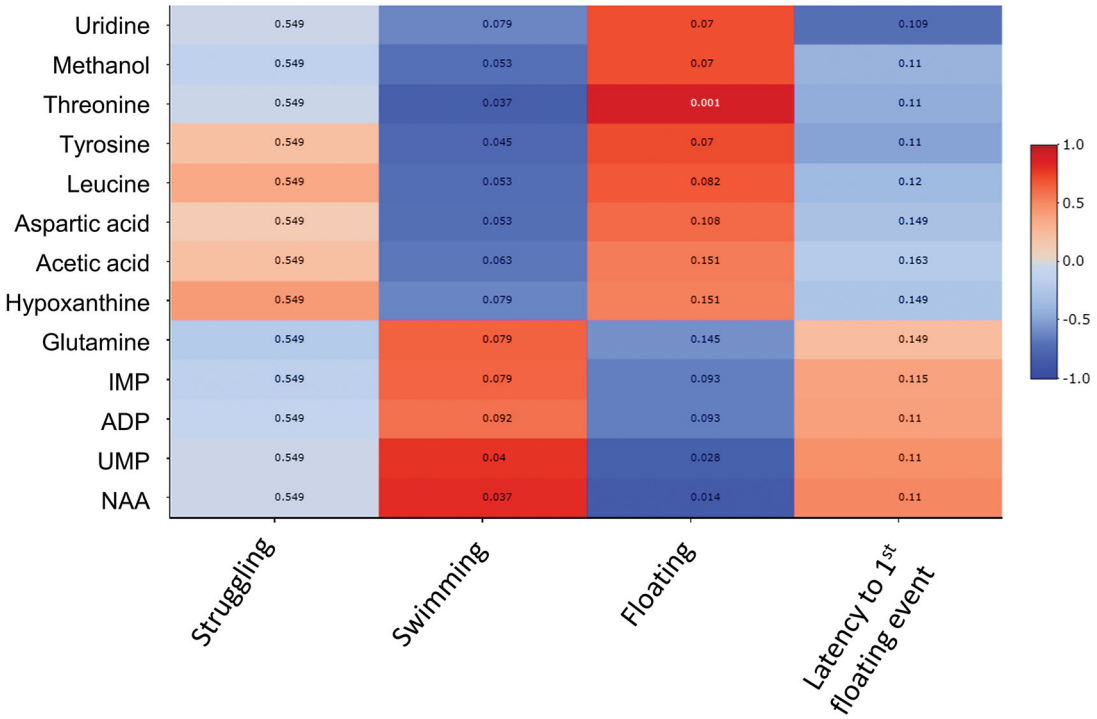

Figure 7. Association of metabolomics data with FST behavioral parameters; struggling, swimming, floating and latency to 1st floating event. Coloring is based on the Spearman's correlation coefficient. The value shown in each cell corresponds to the -$\log_{10}$ (Q-value), where Q-value is the FDR corrected *p*-value from Spearman's correlation. Metabolites with $Q < 0.10$ for at least one behavioral parameter are shown.

cerebellum is connected with stress-related brain regions and processes neurochemical mediators (Rabellino et al., 2018). Cerebellar molecular changes, induced in response to acute or chronic stress, have been observed in animal models. Sabbot and Costin show decreased calcium levels in the cerebellum of male rats (Sabbot & Costin, 1974), while Watanabe et al. report opposite findings in both rats and mice (Watanabe et al., 1987) exposed to a cold environment stress. In patients with cerebellum-confined disorders, impairments of emotional processes have been described, thereby highlighting the role of cerebellar interconnectivity in behavior and higher order functions (Schmahmann & Sherman, 1998). Thus, it has been suggested that the cerebellum may play a role in mood regulation due to its functional and anatomical connections to brain regions central to neuropsychiatric disorders (Konarski et al., 2005). Interestingly, it has been also proposed that dysfunctional neural circuitries connecting cerebellum, cortex and hypothalamus may be responsible for cognitive symptoms in schizophrenia (Andreasen et al., 1996).

We have previously used metabolomic approaches to study neuropsychiatric phenotypes (Filiou et al., 2011, 2014, 2021; Papadopoulou et al., 2019; Zhang et al., 2011) and pertinent treatment responses (Kao et al., 2016; Nussbaumer et al., 2016; Park et al., 2016, 2017; Weckmann et al., 2017, 2019). Metabolomics has been also implemented to study stress effects in the brain, indicating alterations in lipids, amino acids, neurotransmitters and energy-related metabolites (Akimoto et al., 2019; Bassett et al., 2019; Dulka et al., 2017). Here, we report metabolic alterations in the cerebellum of mice after exposure to acute stress and we correlate metabolite levels with behavioral parameters. The most significantly altered metabolic pathway in our analysis was aspartate metabolism, which is implicated in neurotransmission. NAA is synthesized in neurons from aspartic acid and acetyl-coenzyme A, through acetylation by L-aspartate N-acetyltransferase. NAA is degraded by aspartoacylase (ASPA) which cleaves the acetate moiety of NAA to be used in lipid and myelin synthesis in oligodendrocytes responsible for the myelination of the neuronal axons. NAA turnover has been linked to behavioral deficits and neurodegenerative disorders. Region-specific myelin differences have been associated with resilience versus susceptibility upon chronic social defeat stress in mice (Bonnefil et al., 2019). Deletion of an aspartate N-acetyltransferase encoding gene induced behavioral deficits in rodents, including reduced social interaction and altered immobility time in the FST (Toriumi et al., 2015). Decreased NAA levels have been also reported in patients diagnosed with bipolar disorder, Alzheimer's disease and stroke, pointing toward NAA as a marker of brain health (Deicken et al., 2003; Saunders, 2000; Valenzuela & Sachdev, 2001).

NAA also plays a crucial role in mitochondrial function. It is involved in energy production from glutamate, as the acetylation of aspartic acid and its depletion favors the conversion of glutamine to a-ketoglutaric acid, which enters the citric acid cycle in mitochondria (Moffett et al., 2007). NAA biosynthesis is tightly connected to neuronal mitochondrial function with a dependence of NAA synthesis on mitochondrial energy state, which in turn regulates behavioral phenotypes, such as stress and anxiety readouts (Filiou & Sandi, 2019; Picard et al., 2018). NAA concentration has also been associated with ADP levels that may modulate mitochondrial function (Pan & Takahashi, 2005). Here, we observe a significant increase of aspartic acid and acetic acid, with a significant decrease of NAA, indicating reduced NAA biosynthesis and/or its degradation by ASPA. All three metabolites were also associated with FST behavioral parameters (swimming and/or floating), acting as potential markers of response to acute stress. ADP levels were also significantly decreased in the stressed mice, accompanied by a corresponding decrease in NAA levels.

NAA is a precursor of NAAG, the most abundant neuropeptide in the human brain, which modulates release of neurotransmitters, including glutamic acid (Xi et al., 2002). Baslow has proposed that NAA, NAAG and their intermediates, such as acetic acid, aspartic acid and glutamic acid, are cycled between neurons and glia as a mechanism of intercellular signaling (Baslow & Guilfoyle, 2006). The NAAG/NAA ratio has been used as an indicator of brain health in schizophrenia (Jessen et al., 2013). In our analysis, the ratio NAAG/NAA was significantly increased in stressed mice, with NAAG itself being only slightly increased whereas NAA levels being significantly reduced. This change may indicate alterations of the intracellular signaling with no change in overall NAAG levels in the brain. Interestingly, increased levels of GABA, the primary inhibitory neurotransmitter in the brain, have also been observed in our analysis, perhaps in order to counteract the increased glutamatergic activity under stress conditions.

Cerebellum has high energy requirements (Tomasi et al., 2013) leading to the extensive utilization of alternative energy sources when needed (Pellerin, 2003). Perturbed energy metabolism has been reported in the mouse brain under stress (Thurston & Hauhart, 1989). Acetone, one of the main ketones together with acetoacetate and β-hydroxybutyrate, was significantly decreased in the cerebellum of the stressed group. Ketones are produced through fatty acid metabolism under starvation conditions by ketogenesis, being then able to fulfill up to 70% of the energy requirements of the brain (White & Venkatesh, 2011). Several studies have shown that ketogenic diet interventions could exert strong neuroprotective effects on social behavior and cognition (Wlodarek, 2019). Moreover, β-hydroxybutyrate was found to reduce depression- and anxiety-related behaviors in a rodent model of chronic unpredictable stress (Yamanashi et al., 2017). Lactic acid levels, which can be used by the brain as an alternate energy source (Pellerin, 2003; Thurston & Hauhart, 1989), were also increased in the stressed mice.

Purine/pyrimidine metabolism has emerged as an important cerebellar pathway in the discrimination of stressed versus control mice in our study. Purine *de novo* biosynthesis is a complex process with high energy demands, starting with the formation of phosphoribosyl pyrophosphate (PRPP) which leads to the formation of IMP. The latter is subsequently converted to AMP or GMP (Kelley & Andersson, 2014) or can be metabolized to inosine to form hypoxanthine. PRPP is also utilized for the biosynthesis of pyrimidines, beginning with UMP formation. The described pathway summarizing the

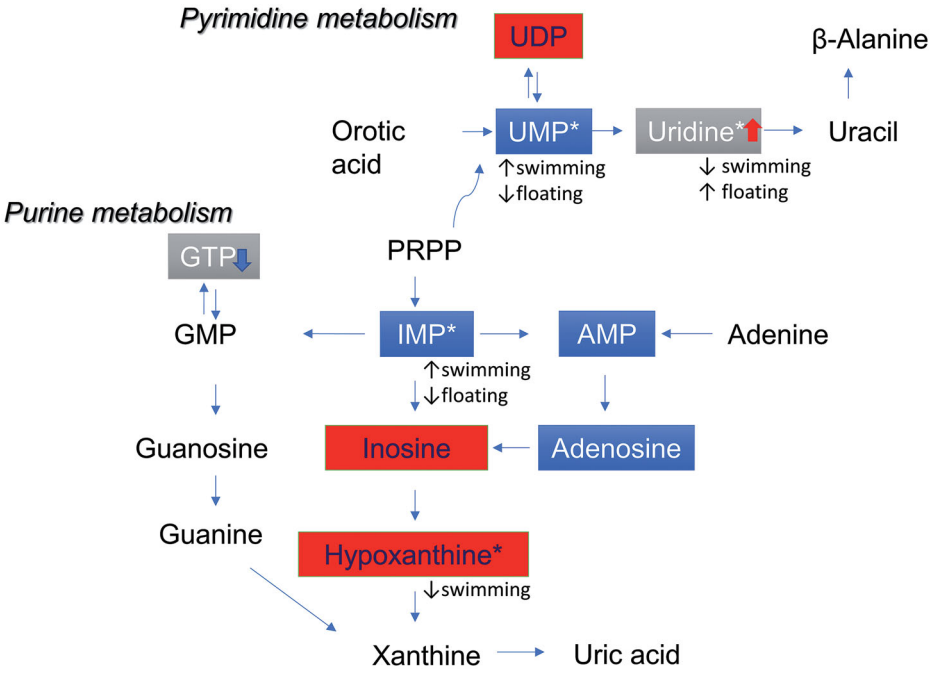

Figure 8. Purine/pyrimidine metabolism alterations in stressed compared to control mice. Metabolites in rectangles have been detected in the NMR analysis. UDP, inosine and hypoxanthine were significantly increased in stressed mice, while UMP, IMP, AMP and adenosine were decreased. Uridine and GTP were not significantly altered (the arrow shows trend for increase or decrease in stressed mice). Metabolites exhibiting significant correlations with behavioral readouts (FDR level at f = 10%) are denoted with (*) and the associated parameters and directionality of effect are shown.

metabolite level alterations for the stressed mouse group is shown in Figure 8. Stressed mice exhibited significantly lower levels of IMP, AMP, UMP and adenosine, and higher levels of inosine, hypoxanthine and UDPs, compared to controls. Remarkably, the correlation analysis of metabolite levels with the behavioral parameters revealed that UDP, IMP and ADP are positively correlated with swimming and inversely with floating in FST. Besides being precursors of nucleic acids, purine and pyrimidine nucleotides are also implicated in a plethora of processes, including energy metabolism, cell growth, metabolic signaling, sugar transport and phospholipid biosynthesis (Fumagalli et al., 2017). The levels of purine and pyrimidine metabolites and their receptors have been associated with various neuropsychiatric disorders and chronic antidepressant treatment response (Park et al., 2016). Moreover, choline and phosphocholines, which act as precursors to membrane phospholipids exhibited a high VIP score in the multivariate analysis. Ethanolamine levels were significantly increased in all analyses suggesting a lipid turnover under stress conditions.

Previous metabolomic studies have examined the metabolome of the hippocampus under stress exposure (Akimoto et al., 2019; Rana et al., 2020). Dulka et al. examined the effects of acute social defeat stress in the metabolome of different brain regions, in order to unravel the underlying mechanisms of stress resilience. Similarly to our findings in the cerebellum, mice susceptible to stress exhibited increased hippocampal GABA levels and decreased IMP and AMP levels in the ventromedial prefrontal cortex compared to stress resilient mice (Dulka et al., 2017). Increased hippocampal GABA levels were also observed as a response to mild or even more intense stressors (de Groote & Linthorst, 2007).

In one of the very few relevant metabolomic studies in the cerebellum, Shao et al. have examined alterations on the cerebellar metabolome in rats exposed to chronic mild stress (Shao et al., 2015) reporting abnormal amino acid metabolism with decreased levels of phenylalanine, tyrosine, lysine, glutamine, and proline and increased levels of glycine in the cerebella of chronically stressed rats. Lower levels of creatinine, and creatinine phosphate, indicated an energy deficiency in the cerebellum of chronically stressed rats. Herein, phenylalanine was found to be significantly upregulated, and glutamine downregulated in the cerebellum of stressed, compared to control mice. Tyrosine levels correlated negatively with swimming. Moreover, stressed mice exhibited lower creatinine levels in the PLS-DA analysis (Figure 5(C)), suggesting an energy deficiency compared to control mice.

In this study, different metabolomics data analysis methods were implemented, including univariate and multivariate approaches as well as correlation with behavioral readouts, in order to thoroughly compare stressed versus unstressed cerebella. Univariate analysis highlights which of the identified metabolites exhibited significant alterations, while multivariate analyses allowed for the "metabolic fingerprinting" of the two groups, incorporating all spectral information and taking advantage of the global nature of untargeted metabolomics. We performed both the unsupervised PCA, which is a data reduction method to identify clusters of samples that share common variation as well as possible outliers, and the supervised PLS-DA and OPLS-DA methods, which utilize class information and focus on group differentiation. With supervised approaches, clearer separations are often observed; however, prior class information introduces bias into the analysis. On the other hand, in the PCA analysis the intra- should be

much larger than the inter-group variation for clear separations to be obtained (Worley and Powers, 2013). Since alterations on cerebellar metabolome after stress exposure are largely unknown, the use of PCA initially offered an exploratory view on the relationships between the two groups, as well as an unbiased confirmation of some kind of differentiation. Subsequent PLS-DA or OPLS-DA analyses led to optimized separations, enabling a more detailed examination of the observed differences. Finally, OPLS-DA models compared to PLS-DA, have the advantage of eliminating variation in the metabolomics data which is unrelated to the stress variable.

FST as an acute stressor entails an activity component (i.e. swimming, struggling), therefore a contribution of physical activity on the observed metabolic alterations cannot be unequivocally excluded. In our efforts to address this, we have introduced a recovery interval of up to 2 h after FST so as to explore persistent stress effects rather than short-term effects of mobility-related activity. Further investigations using an additional stressor with a minimal activity component such as acute restraint stress and/or including a control group with a physical component which may not be perceived as unpleasant, such as voluntary physical activity (e.g. running wheel), will be needed to consolidate our findings and confirm that the observed differences can be exclusively attributed to the experience of stress. Future studies should also highlight divergent metabolomic signatures between acute and chronic stress in the cerebellum.

Conclusions

Taken together, the metabolic response induced in the mouse cerebellum by an FST stressor was investigated, in an effort to understand the biochemical changes induced by acute stress in this brain region. Using metabolomics, we were able to discriminate stressed from control mice, highlighting alterations in glutamatergic and GABAergic neurotransmission and major metabolic pathways (energy and purine/pyrimidine metabolism) as well as correlating individual metabolite levels with FST parameters. Our data pave the way to investigate stress-related mechanisms in the cerebellum, and highlight the need to examine understudied brain areas in the context of emotional processing in order to gain an understanding of brain stress responses and pertinent pathobiology.

Acknowledgments

We thank Dr. Panagiotis Lekkas and the personnel of the University of Ioannina Animal Facility for expert animal care.

Author contributions

AI: formal analysis, investigation, visualization, writing – original draft; writing – review & editing; AMV: investigation, methodology, visualization, writing – original draft; writing – review & editing; MN: formal analysis, methodology, visualization, writing – review & editing; DB: formal analysis, methodology, visualization, writing – review & editing; EM: methodology, supervision, writing – review & editing; EG: conceptualization, funding acquisition, project administration, supervision, writing – review & editing; MDF: conceptualization, funding acquisition, project administration, supervision, writing – original draft, writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research is co-financed by Greece and the European Union (European Social Fund-ESF) through the Operational Programme "Human Resources Development, Education and Lifelong Learning 2014–2020" in the context of the project "How do mitochondria regulate stress? Let's ask metabolomics" (MIS 5047652).

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