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Behavioral deficits, abnormal corticosterone, and reduced prefrontal metabolites of adolescent rats subject to early life stress

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HIGHLIGHTS

- Early life stress induced depressive-like behavior in adolescent rats.
- Early life stress increased corticosterone level in adolescent rats.
- Early life stress reduced prefrontal glutamate, glutamine, and N-acetylaspartate.
- The findings underscore the long-lasting and detrimental effects of childhood adversities.

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ABSTRACT

The present study investigated the effect of early life stress in adolescent rats on brain metabolites, serum corticosterone, and depressive-like behavior. A group of rats was subject to early life stress from postnatal day (PND) 1 to 14. A matched control group was studied. Behavioral tests, serum corticosterone and high-resolution proton magnetic resonance spectroscopy were conducted between PND 30 and 40. In this study, adolescent rats exposed to early life stress demonstrated depressive-like behavior and increased serum corticosterone during adolescence. They also showed reduced glutamate, glutamine, and N-acetylaspartate (NAA) levels in the prefrontal cortex. A reduced myo-inositol level, consistent with astroglial deficits, was observed but was not statistically significant. Together, these findings characterize the effect of early life stress on adolescent animals and underscore the long-lasting and detrimental effects of childhood adversities.

1. Introduction

Major depressive disorder is a debilitating mental illness and worldwide leading cause of disability [31]. Environmental risk factors, especially early life stress, appear to play a pivotal role in the pathophysiology of major depression [12]. Therefore, investigating the neurobiological mechanisms of early life stress is essential for a better understanding of major depression, and perhaps other stress-related psychiatric disorders [12]. Clinical studies have associated history of child abuse with abnormal neuroendocrine responses and increased risk of major depression [11]. Moreover, the prefrontal cortex and the hippocampus, two brain regions implicated in stress response, were found to have reduced volume in adults exposed to early life stress [3,35]. Consistent with these human findings, preclinical evidence from both rodents and nonhuman primates showed behavioral deficits with long-term neurobiological alterations induced by early life stress [7,22]. Thus, early life stress in animals provides a unique model for investigating the neurodevelopmental mechanisms of major depression.

Adolescence is a vital phase of development demonstrating high neuroplasticity and sensitivity to early adverse events [21]. Several neuropsychiatric disorders, including major depression, may first present during adolescence [1,25]. Yet, the effects of early adversities have not been extensively studied in adolescent animals. A

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study by Ichise et al. reported abnormalities in the serotoninergic system of peer-reared non-human primates adolescents [15]. However, the developmental effects of early stress on the glutamatergic system are not well understood.

The aim of the current study was to examine whether early life stress will induce depressive-like behavior and alter the glutamatergic system in adolescent rats. For this purpose, we used proton magnetic resonance spectroscopy (^{1}H MRS) to quantify the level of glutamate and other brain metabolites in the prefrontal cortex. The prefrontal cortex was selected as the brain region of interest given its crucial role in emotional regulation and stress response [17]. Metabolites measured by ^{1}H MRS included: glutamate, glutamine, N-acetylaspartate (NAA), choline compounds (Cho), myo-inositol, and creatine (Cr). For detailed description of the physiologic function of these metabolites please see the recent review by Maddock and Buonocore [20]. Briefly, glutamate and glutamine are an amino acid neurotransmitter and its derivative, respectively. Their levels may reflect local neuronal activity. NAA, the most abundant neuronal amino acid, is commonly used as a marker of neuronal integrity. Cho increases have been interpreted to reflect increased cell membrane turnover, and myo-inositol was associated with astroglial metabolism. Cr increases have been associated with increased myelination, however, Cr tends to be stable in the absence of major pathology [20]. Prior ^{1}H MRS studies in neonatal [19] and adult [13,14] animals exposed to early life stress have reported altered levels of glutamate, NAA, Cho, and myo-inositol. The current study extends prior evidence to investigate the developmental effect of early life stress on prefrontal metabolites in adolescent rats.

2. Materials and methods

2.1. Animals

Parental Sprague-Dawley rats were purchased from Animal Center of Shantou University Medical College and mated (one male with two females) in Shantou University Mental Health Center animal facility two weeks after their arrival. A total of 32 rats were studied. The early life stress group had 8 males and 8 females. Similarly, the control group had 8 males and 8 females. All animals were housed in a temperature ($22 \pm 1\,^\circ$C) and humidity ($55 \pm 4\%$) controlled room on a 12-h light/dark cycle (lights on at 07:00 a.m.) with food and water provided *ad libitum*. All experimental procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Laboratory Animals Care and Use committee of Shantou University Medical College.

2.2. Early life stress

The early life stress protocol was conducted as previously described [5]. Briefly, dams were first removed and pups were separately placed in a plastic container with bedding from the fostering cage. Pups were moved to an adjacent room keeping constant temperature with 34 °C during separation period. The separation procedure was conducted for 180 min (08:30 a.m. and 11:30 a.m.) from PND 1 to 14. After separation, the pups and dams were reunited at the maternity cages. Animals in the control group were left with their mothers undisturbed between PND 1 and 14, with the exception of the weekly cage cleaning.

2.3. Behavioral tests

All behavioral tests took place during adolescence between PND 30 and 38 [18]. The sequence of behavioral tests was based on our previous report [16]. Sucrose preference and forced swimming tests were selected given their relevance to depression symptoms of anhedonia and despair, respectively [24].

2.3.1. Sucrose preference test

Sucrose preference test was conducted as previously described [16], with minor modification. In summary, single-housed animals were habituated to 1% sucrose solution for two days followed by 24 h food and water deprivation. Then, animals were exposed for 3 h to two identical bottles, one filled with water and the other with 1% sucrose. The position of bottles was counterbalanced across the left and right side of the test cages. Sucrose preference was calculated as follow: sucrose consumption percentage = sucrose consumption/(sucrose consumption + water consumption) × 100.

2.3.2. Forced swimming test

Forced swimming test was performed according to previous reports [23,28]. Rats were placed individually in a transparent cylindrical tank (30 cm in diameter, 50 cm height) containing water at 23–24 °C with a 30 cm depth. All animals were exposed to 15 min swimming, 24 h prior to the 5 min swimming test. Immobility time (rats floated without struggling or making least movements necessary to keep their heads above the water) was scored from videotapes by two trained observers who were blind to the experimental conditions. The agreement between the two observers was substantial with a kappa coefficient = 0.65.

2.4. Serum level of corticosterone

Trunk blood samples were collected at PND 40 between 10 a.m. and 12 p.m. Serum was taken after blood samples centrifuged at $1200 \times g$ for 30 min, then frozen at −80 °C. Serum corticosterone levels were measured using Corticosterone ELISA kit (Abcam LLC, Cambridge, UK). The range of detectable level is 20–400 ng/ml.

2.5. ^{1}H MRS acquisition and processing

At PND 40, animals were rapidly decapitated without anesthesia. The whole brain was rapidly removed from the skull and put on dry ice immediately. Olfactory bulbs were firstly removed followed by cutting out bilateral prefrontal cortex according to a rat brain atlas [26], which included the anterior cingulate, infralimbic, prelimbic, and ventrolateral orbitofrontal cortices. The dissected region coordinates were as follow: Anterior – 4.2 mm anterior to bregma, 2.4 mm lateral to midline, 2.2 mm ventral to the skull; Posterior – 2.2 mm anterior to bregma, 2.4 mm lateral to midline, 5.5 mm ventral to the skull. Additional detail on prefrontal cortex of rat can be found in Ref. [34]. The bilateral prefrontal cortices – and other brain regions (not reported here) – were stored at −80 °C until analysis. The preparation of the brain samples was based on our previous report [4]. Frozen brain tissue was homogenized with pestle mixed with 0.5 M ice-cold perchloric acid for 15 min on dry ice. The homogenate was centrifuged at $10{,}000 \times g$ for 20 min at 4 °C. The supernatant was neutralized with 1.0 M KOH and lyophilized for about 36 h. Dried extracts were suspended in 500 μl D_2O containing 2,2′-3,3′-tetradeutero-trimethyl-sylilpropionate (TMSP), and then transferred into 5 mm nuclear magnetic resonance tubes for ^{1}H MRS detection.

All ^{1}H MRS data were acquired on BrukerAvance 400 MHz high-resolution (9.4 T) magnetic resonance spectrometer (Bruker Co., Germany). Free induction decays (FIDs) were collected on 4096 data points over a spectral width of 5000 Hz with a relaxation delay of 5 s and the number of scans was 128. Spectra processing included Fourier-transformation, phase correction and baseline correction, using XWINNMR (Bruker GmBH). The calculation of metabolites concentration was based on previous reports [4,10]. The chemical shifts were assigned according to the external

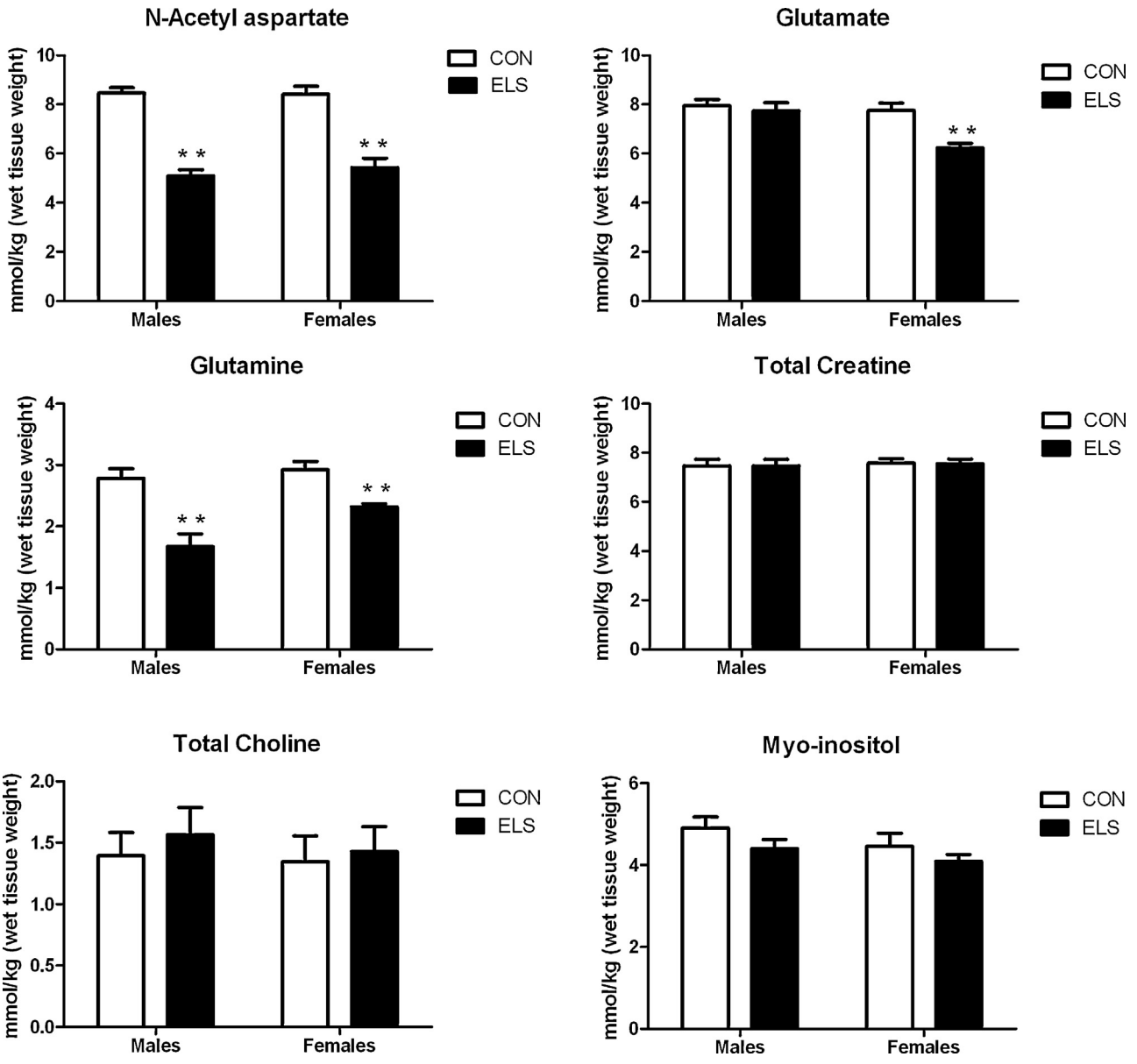

Fig. 1. Effect of early life stress on the prefrontal cortex metabolites in adolescent rats. The data are presented as mean ± standard error (S.E). $^{**}p < 0.01$ compared to the same sex CON. Abbreviations: CON, control group; ELS, early life stress group.

standard TMSP and analyses were then performed using MestRe-c 4.3 (http://qobrue.usc.es/jsgroup/MestRe-c) within a spectral window ranging from 1.0 to 4.2 ppm (Fig. 1). TMSP was used as the external reference and concentration of each metabolites was calculated according to the formula followed: metabolites concentration = (integral values of metabolite/integral values of TMSP) × (number of protons of metabolite/9) × concentration of TMSP (mmol/L). To account for variations in the mass of individual samples, absolute concentrations of MR metabolites were normalized to tissue weight and are expressed as mmol/kg of wet tissue weight.

2.6. Statistical analysis

Statistical analyses were conducted using SPSS (19.0.0 IBM SPSS Inc.). All data were analyzed using two-way ANOVA, with group (control or stress) and sex (male or female) as independent factors. Bonferroni multiple comparisons test was used for *post hoc* analysis. Associations between all outcome measures were explored using Pearson's correlation. The inter-rater reliability for immobility time of FST was measured with kappa coefficient. All tests were two-tailed, with significance level set at $p = 0.05$. Values are expressed as mean ± standard error (S.E).

3. Results

3.1. Effect of early life stress on behavioral tests in adolescent rats

3.1.1. Sucrose preference test

Two-way ANOVA revealed a significant group effect [$F_{(1,28)} = 36.2$, $p < 0.001$] and sex effect [$F_{(1,28)} = 4.9$, $p = 0.04$], but no group-by-sex interaction ($p = 0.30$). *Post hoc* comparisons indicated that early life stress reduced sucrose preference in females [control mean ± S.E. = 90 ± 3 to stress mean ± S.E. = 64 ± 4, $F_{(1,28)} = 12.3$, $p = 0.002$] and males [control mean ± S.E. = 83 ± 6 to stress mean ± S.E. = 46 ± 8, $F_{(1,28)} = 25.0$, $p < 0.00$] (Fig. S2-A).

3.1.2. Forced swimming test

Two-way ANOVA revealed a significant group effect [$F_{(1,28)} = 40.1$, $p < 0.001$], but no sex effect ($p = 0.36$) or group-by-sex interaction ($p = 0.20$). *Post hoc* comparisons indicated that early life stress increased immobility time in females [control mean ± S.E. = 22 ± 5 to stress mean ± S.E. = 68 ± 9, $F_{(1,28)} = 29.8$, $p < 0.001$] and males [control mean ± S.E. = 24 ± 5 to stress mean ± S.E. = 54 ± 4, $F_{(1,28)} = 12.8$, $p = 0.001$] (Fig. S2-B).

3.2. Effect of early life stress on serum corticosterone level in adolescent rats

Two-way ANOVA revealed a significant group effect [$F_{(1,28)} = 34.7$, $p < 0.001$], but no sex effect ($p = 0.86$) or group-by-sex interaction ($p = 0.35$). *Post hoc* comparisons indicated that early life stress increased serum corticosterone in females [control mean ± S.E. = 185 ± 9 to stress mean ± S.E. = 243 ± 11, $F_{(1,28)} = 12.2$, $p = 0.002$] and males [control mean ± S.E. = 172 ± 13 to stress mean ± S.E. = 252 ± 14, $F_{(1,28)} = 23.4$, $p < 0.001$] (Fig. S3).

3.3. Effects of early life stress on the prefrontal cortex metabolites in adolescent rats

Means and S.E. are depicted in Fig. 1. Two-way ANOVA revealed a significant group effect on NAA [$F_{(1,28)} = 118$, $p < 0.001$], glutamate [$F_{(1,28)} = 9.8$, $p = 0.004$], and glutamine [$F_{(1,28)} = 32.9$, $p < 0.001$]. Sex effect was significant for glutamate [$F_{(1,28)} = 9.5$, $p = 0.005$] and glutamine [$F_{(1,28)} = 6.8$, $p = 0.01$], but not NAA ($p = 0.63$). Group-by-sex interaction was significant for glutamate [$F_{(1,28)} = 5.5$, $p = 0.03$], but not NAA ($p = 0.52$) and glutamine ($p = 0.10$). *Post hoc* comparisons indicated that early life stress reduced NAA and glutamine in both females and males ($p < 0.05$). However, the effect of early stress on glutamate was evident in females only ($p = 0.001$), but not males ($p = 0.60$). Two-way ANOVA showed no significant ($p > 0.05$) group, sex, or group-by-sex effects on Cr, Cho, and myo-inositol (Table S1 in Supplements).

3.4. Correlation analysis between behavioral and biological measures

Within each group (control or stress), an exploratory analysis was conducted to correlate behavioral measures (sucrose preference and immobility time) with biological measures (corticosterone, NAA, glutamate, glutamine, Cr, Cho, and myo-inositol). We found a significant correlation between serum corticosterone and immobility time in the stress group ($r = -0.61$, $n = 16$, $p = 0.01$), but not in the control ($r = 0.22$, $n = 16$, $p = 0.42$) (Fig. 2). Fisher r-to-z was used to compare these two correlation coefficients ($r = -0.61$ *vs.* $r = 0.22$), which were found to be significantly different between groups ($z = -2.38$, $p = 0.02$). No other statistically significant correlations between behavioral and biological measures were found ($p > 0.05$).

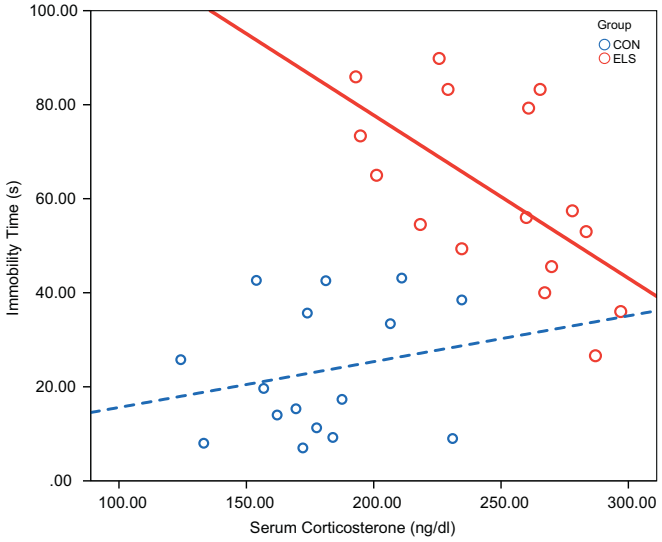

Fig. 2. Correlations between serum corticosterone and immobility time during forced swim test in control (blue: $r = 0.22$, $n = 16$, $p = 0.42$) and stressed (red: $r = -0.61$, $n = 16$, $p = 0.01$) adolescent rats. These two correlation coefficients were significantly different (Fisher r-to-z: $z = -2.38$, $p = 0.02$). CON, control; ELS, early life stress. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

In this study, adolescent rats exposed to early life stress showed reduced glutamate, glutamine, and NAA levels in the prefrontal cortex. Myo-inositol, a putative marker of astroglial function [2], was numerically reduced yet not statistically significant ($p = 0.09$). The effect of early stress on glutamate was limited to females. Early life stress animals showed depressive-like behavior and increased serum corticosterone during adolescence. Of interest, in the stress group we found a negative correlation between immobility time and corticosterone levels. This preliminary finding suggested that stress adolescent rats with increased corticosterone were more resilient to despair as measured by immobility time during the forced swim test paradigm. However, it is important to be cautious when interpreting the latter finding, given the exploratory nature of this analysis. In particular, this correlation analysis was conducted without *a priori* hypothesis; as such the finding will not survive a conservative Bonferroni correction for multiple comparisons (adjusted $p = 0.05/28$).

Studies investigating the long-term effects of early life stress have consistently shown depressive-like behavior in adult animals [8,14]. However, the effects of early adversities on adolescent animals were not fully characterized. The present study contributes to a better understanding of the effect of early stress on adolescents. We found that the behavioral and neurobiological deficits, induced by early stress, are present during adolescence. Moreover, our finding of increased corticosterone is consistent with accumulating evidence associating early life stress with lifelong HPA axis dysfunction [6,21]. Of note, one previous early life stress study found reduced corticosterone levels in adolescent females but no difference among males [18]. In contrast, we observed robust increase of corticosterone in both genders. The difference in stress duration and timing may have accounted for the discrepant results.

Convergent evidence has implicated the glutamatergic system in the pathophysiology of early life stress [22,29] and stress-related neuropsychiatric disorders [32]. Magnetic resonance spectroscopy has been successfully used to examine the effect of early stress on adult animals. Hui et al. found reduced glutamate/Cr, NAA/Cr, and myo-inositol/Cr in the hippocampus of rats subject to early life stress [14]. Similarly, Llorente et al. recently reported reduced Glx, a composite measure of glutamate and glutamine, in the hippocampus and the prefrontal cortex of maternally deprived rats [19]. Of interest, antidepressants and environmental enrichment opposed the effects of early life stress on these metabolites [13,14]. In the current study, instead of measuring glx, the use of high-resolution (9.4 T) ^{1}H MRS allowed us to have well resolved glutamate and glutamine spectral signals, and permitted the absolute quantification of glutamate and glutamine. Consistent with previous studies [14,19], we found reduced glutamate, glutamine, and NAA in adolescent rats subject to early life stress. Of interest, male adolescents were resilient to the effect of early life stress

on prefrontal glutamate, as such the stress-induced reduction in glutamate was limited to female adolescents.

The clinical implication of our rodent findings stems from extensive evidence implicating the glutamatergic system and neuroplasticity in the neurobiology of major depression [33]. It has been postulated that HPA axis malfunction precipitating altered glutamate release – combined with astroglial deficits impairing glutamate uptake – results in increased extracellular glutamate and subsequent excitotoxicity [27,32]. Consistent with this hypothesis of depression, repeated evidence demonstrated reduced glutamate and NAA levels in the frontal lobe of patients with major depression, although not without inconsistency [9,36]. Similarly, in our study we found long-lasting effect of early adversity on the prefrontal cortex glutamate and NAA. Finally, it is well documented that, compared to men, women are at increased risk for major depressive disorder. Therefore, our finding of glutamate alterations in females raises the question if developmental glutamate abnormalities during adolescence confer a risk for adult major depression. Carbon-13 spectroscopy is a unique technique to investigate the glutamatergic synapse *in vivo* both in animals and in humans [30]. Future studies employing *in vivo* ^{1}H MRS and ^{13}C MRS methods combined with longitudinal design are needed to investigate the role of developmental glutamate in adult major depressive disorder.

In summary, our results indicate that adolescent rats subjected to early life stress have depressive-like behavior, high serum corticosterone, and reduced levels of prefrontal glutamate, glutamine and NAA. A reduced myo-inositol level, consistent with astroglial deficits, was observed but this was not significant. Together, these findings characterize the effect of early life stress on adolescent animals and underscore the long-lasting and detrimental effects of childhood adversities. Finally, a limitation of the current report is the lack of data on the effect of early life stress on the hippocampus and amygdala, two brain regions implicated in emotion regulation and stress response. Future studies are needed to extend our findings and to investigate other brain regions.

Conflicts of interest

The authors report no biomedical financial interests or potential conflicts of interest.

Contributors

Renhua Wu, Yaowen Chen and Jie Zhang designed the study and wrote the first draft of the paper. Yaowen Chen, Tianhua Huang, Jie Zhang, and Chadi G. Abdallah conceptualized the MRS study and contributed to the interpretation of the data. Qingjun Huang, Chongtao Xu, Jie Zhang, Yeyu Xiao, Yan Ding, and Yuzhen Liu, performed animal behavior test and serum corticosterone determination. All authors contributed to and have approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2013.04.035.

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