

Cerebral Metabolic Changes in a Depression-like Rat Model of Chronic Forced Swimming Studied by Ex vivo High Resolution ^1H Magnetic Resonance Spectroscopy

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Abstract Many previous in vivo ^1H magnetic resonance spectroscopy (MRS) studies have shown that patients with major depressive disorder (MDD) are associated with perturbations of cerebral metabolism of neurotransmitters glutamate (Glu) and γ -aminobutyric acid (GABA). In this study, we investigated the changes of cerebral metabolism in a depression-like rat model of chronic forced swimming stress (CFSS). The aims are to further understand the pathophysiological mechanisms underlying CFSS treatment, and to further establish the face and predictive validity of the CFSS model. The results showed that, relative to control, the CFSS rats had significantly reduced Glu, taurine and glutamate + glutamine (Glx) levels in the PFC, and significantly reduced N-acetyl aspartate (NAA) level, Glu level and Glu/GABA ratio in the hippocampus.

Taking together, these results suggest that CFSS treatment can induce region-specific changes in the metabolism of Glu. The CFSS model might be used to study antidepressants specifically targeting the central glutamatergic system.

Keywords Depression · Chronic forced swimming · NMR · Glutamate · Metabolism · Rat

Introduction

A growing body of evidence, mainly derived from in vivo ^1H magnetic resonance spectroscopy (MRS) studies, has shown that patients with major depressive disorder (MDD) are associated with perturbations of cerebral metabolism of excitatory neurotransmitter glutamate (Glu) and inhibitory neurotransmitter γ -aminobutyric acid (GABA) [1, 2]. The brain regions implicated include prefrontal cortex (PFC) [3–5], occipital cortex [6, 7], anterior cingulate cortex [8, 9] and amygdala [10] etc. In addition, Michael et al. found that the total level of Glu and glutamine (Gln; Glx: Glu + Gln) in the left dorsolateral PFC of the depressed patients is significantly reduced compared to the control, and it correlates negatively with severity of depression [5]. It has also been shown by Hasler et al. that unmedicated MDD patients have significantly reduced Glx and GABA levels in a dorsomedial/dorsal anterolateral prefrontal region, and significantly reduced Glx level in a ventromedial prefrontal region [4].

Chronic forced swimming stress (CFSS) model, developed from the well-known forced swimming test (FST) [11], is an animal model stimulating the core symptom of behavioral despair observed in human depression patients. The rodent model of CFSS has been widely applied for

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studying the physiopathology of depression and for screening antidepressants [12–15]. However, most of the previous studies on the CFSS model were focused on the central serotonin (5-HT) system [12, 15]. Few have investigated whether the central glutamatergic and GABAergic systems are affected or not in this model.

In this study, we investigated the changes of cerebral metabolism in bilateral PFC and hippocampus of rats subjected to CFSS by *ex vivo* high resolution ^1H MRS, and correlated the findings to the results of behavioral and histological evaluations. The purpose of the study is to investigate whether cerebral metabolic changes observed in the CFSS rats, if any, resemble those found in the MDD patients. The results would be helpful to further understand the pathophysiological mechanisms underlying CFSS treatment, and to further establish the face and predictive validity of the CFSS model [16].

Materials and Methods

Animal Preparation

All animal preparation procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Wistar rats (6–8 weeks old, weighing 180–200 g) were purchased from Tongji Medical College Experimental Animal Center, Huazhong University of Science and Technology, and maintained in ABSL-3 Laboratory, Experimental Animal Center, Wuhan University, with free access to food and water.

The experimental protocols are depicted schematically in Fig. 1. A 7-day adaptation period was applied to all the animals before the experiments. Behavioral tests, including a 5-min open field test (OPT) and a 5-min FST, were performed to screen for animals showing atypical behaviors and to establish the baseline conditions of the animals being included into the study. The animals meeting the inclusion criteria (see below) were randomly assigned into two groups, the control group (Con, $n = 12$) and the 2 weeks CFSS group (CFSS, $n = 12$). The rats in the CFSS group underwent a session of 15-min forced swimming once daily for 14 consecutive days. All rats were weighted daily. For each group, half of the animals were used for MRS measurements and the other half for histological assessments.

Behavioral Tests

Each rat subjected to CFSS and FST was placed in a water tank (80 cm length \times 60 cm width \times 40 cm height) maintained at a temperature of either 25°C (for CFSS) or 30°C (for FST) [17]. The water was deep enough (i.e., 35 cm) so that the animals could swim or float without their tails touching the bottom. Water was changed for different animals. The animals were forced to swim uninterruptedly for 5 min and 15 min, respectively, in the FST and CFSS sessions. The swimming behaviors of the animals in the FST were recorded by a video camera positioned on the top of the swimming tank. After swimming, the animal was dried and returned to its home cage.

The swimming behaviors of the rats in FST were evaluated by a single observer. The time during which the animals exhibited one of the following three types of behaviors were counted and then added separately (1) immobility: floating in the water without struggling, and making only those movements necessary to keep the head above the water; (2) swimming: making active motions throughout the swim tank and crossing into another quadrant; (3) climbing: making active movements with forepaws in and out of the water, usually upward-directed against the walls [18]. The rats found to have immobility time, swimming time, or climbing time significantly deviated from the group mean (i.e., beyond mean \pm 2 standard deviation (SD)) under baseline conditions was excluded from further experiments. For the animals included in the study, FST and OPT were performed again after the 14-day treatment period (Fig. 1).

The OPT test was referenced from the procedures of Grønli et al. [19]. In brief, each rat was placed at the center of a black-colored square-shaped floor (i.e., 100 cm \times 100 cm) surrounded with 40-cm high cardboard walls. The ground was divided into 25 squares having equal areas by white-colored grids. The spontaneous locomotive behaviors of rats on the floor were recorded by a video camera for a period of 5 min. The number of squares crossed (i.e., at least three paws passing on the same square) and the number of rearing (i.e., posture sustained with only hind-paws on the floor) were counted. The floor was wiped thoroughly after each trial.

NMR Experiments

The rats were sacrificed by decapitation at 48 h after receiving their last forced swimming session. Specimens of

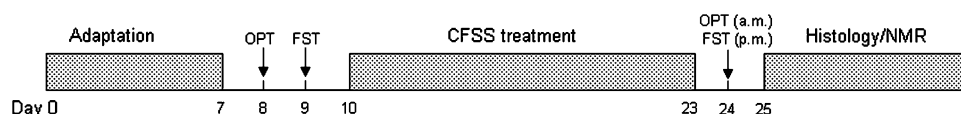


Fig. 1 A schematic representation of the experimental protocols used in this study. OPT: open field test, FST: forced swimming test

bilateral PFC and entire hippocampus formation were immediately dissected, snap-frozen in liquid nitrogen and stored in a -80°C refrigerator until use. For each rat, the PFC sample dissected might include cingulate cortex, prelimbic cortex, infralimbic cortex, ventral orbital cortex, lateral orbital cortex, medial orbital cortex, claustrum, and small parts of secondary motor cortex and primary motor cortex.

The frozen tissues were weighted and ground in a mortar cooled in liquid nitrogen. Ice-cold perchloric acid solution (e.g., 12% (vol/vol), ~ 1.0 ml for the PFC and ~ 0.5 ml for the hippocampus, depending on the amount of the sample) was added into the mortar drop by drop, and the frozen mixture was ground again. The pulverized mixture was then transferred to a Dounce homogenizer and homogenized at 4°C after thawing. The homogenate was centrifuged at 10,000 g for 20 min at 4°C . The supernatant was extracted, neutralized with 1.0 M KOH, centrifuged again to eliminate perchlorate salts, and lyophilized for about 36 h. The dried metabolite mixture was weighted and then dissolved in 0.6 ml of 99.5% D_2O for NMR spectroscopy.

All ^1H NMR experiments were carried out on a Varian INOVA-500 spectrometer at 25°C . A pulse-acquisition sequence was used with a 90 degree flip angle, a 12 s relaxation delay to ensure full relaxation, a spectral width of 8,000 Hz, 32 k data points and 256 averages. The residual water signal was suppressed by homonuclear presaturation with a duration of 3 s. The spectra were zero-filled to 64 k, corrected manually for phase and baseline and referenced to the chemical shift of trimethylsilyl-propionic-2, 2, 3, 3- d_4 -acid (TSP) methyl peak at 0 ppm. Peak area integration was performed using the standard routines provided by the XWINNMR software package (version 2.1, Bruker).

Using the concentration of total creatine (creatine + phosphocreatine, Cr) as the internal reference (8 mmol/kg tissue) [20, 21], the concentrations of metabolites were determined from the spectra and normalized to the weight of the freeze-dried metabolite mixture. Absolute metabolite concentrations in the unit of mmol/kg wet tissue weight were also calculated for the control rats.

Histological Assessments

The rats were deeply anesthetized with an overdose of 5% chloral hydrate and perfused transcardially first with 200 ml saline and then with 350 ml 4% paraformaldehyde in phosphate-buffered solution (PBS) at pH 7.4. Brains were immediately removed and stored in the same fixative overnight at 4°C until being sectioned on a cryostat (Leica, Germany). Coronal brain sections were cut at a thickness of either 25 μm [22] (i.e., for glial fibrillary acidic protein (GFAP) immunohistological chemistry) or 10 μm (i.e., for Nissl staining).

For GFAP staining, free-floating brain sections were first washed with PBS and then incubated with 0.2% Triton X-100 for 30 min at 37°C . Following four rinses with PBS, the sections were blocked with 10% normal goat serum to suppress nonspecific background staining, followed by incubation with the primary antibody purchased from Zymed (Beijing, China) first at 37°C for 2 h, and then at 4°C for 60 h. Bound antibodies were detected using the Histostain SP kit purchased from Zymed (Beijing, China) with diaminobenzidine as the substrate. For Nissl staining, the brain sections were transferred onto glass slides coated with polylysine. The slides were then incubated with 5% toluidine blue at room temperature for 15 min. Following rinses with tap water, the sections were dehydrated and mounted.

The brain sections were examined under an Olympus BX60 microscope. The locations of the regions of interest (ROIs) were first determined at low amplification power ($40\times$). Further analyses were carried out under higher ($200\times$ or $400\times$) amplification power. The number of GFAP-immunoreactive (GFAP-IR) astrocytes and Nissl-positive neurons in the sub-fields of the hippocampus (i.e., CA1, CA2, CA3, CA4 and dentate gyrus (DG)) and four different sub-regions of PFC (cingulate cortex, prelimbic cortex, ventral orbital cortex and medial orbital cortex) were counted and then converted into cell densities. A single visual field was used for CA2, CA3, CA4 and sub-regions of PFC, while multiple (i.e., 3–5) non-overlapped visual fields were used for CA1 and DG. The averaged densities of positively-stained cells in each ROI were considered to be representative of the level of immunoactivity in the region.

Statistical Analysis

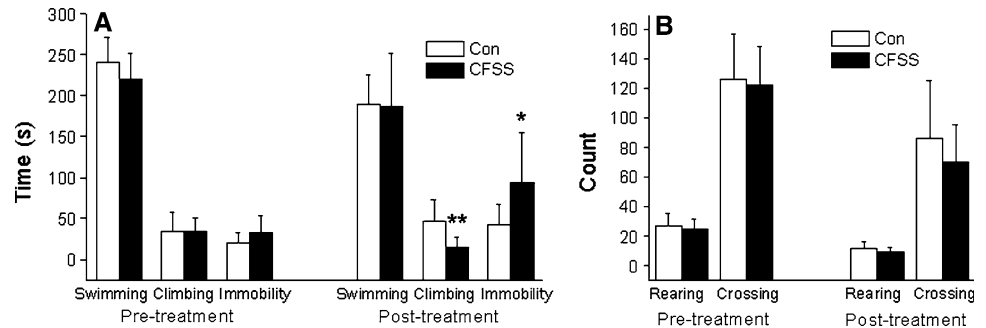
Two-sample *t* tests were used to compare inter-group differences in body weight gains, behavioral scores, metabolite concentrations/concentration ratios and immunoactivities. A $P < 0.05$ was considered to be statistically significant.

Results

CFSS-induced Behavioral Changes

No statistically significant inter-group differences were found in any of the behavioral scores and body weight under baseline conditions. After the 14-day treatment, the CFSS rats showed significantly reduced climbing time ($P < 0.01$) and significantly increased immobility time ($P < 0.05$) in the FST, relative to the control rats (Fig. 2A). The CFSS treatment did not result in any statistical significant

Fig. 2 The effects of CFSS on the scores of the forced swimming test (A) and the open field test (B). The results are expressed as mean \pm SD with $n = 6$ for each group. * $P < 0.05$ and ** $P < 0.01$ relative to the control group (Con)



behavioral changes in OPT (Fig. 2B). No statistically significant inter-group differences were found in body weight gains during the 14-day period.

CFSS-induced Metabolic Changes

Figure 3 shows representative ^1H NMR spectra of the PFC extracts obtained from rats in the control group and the CFSS group. Spectral assignments presented in Fig. 3 are based on literature results. The inlet shows the location where the PFC samples for NMR analysis were taken. Metabolite concentrations and concentration ratios in the PFC and hippocampus are plotted in Fig. 4A and B. Compared to the control rats, the CFSS rats showed significantly reduced Glu ($P < 0.05$), taurine (Tau, $P < 0.05$) and Glx ($P < 0.01$) levels in the PFC. The N-acetyl aspartate (NAA) ($P < 0.05$) and Glu ($P < 0.05$) levels and the Glu/GABA ratio ($P < 0.05$) in the hippocampus of the CFSS rats were significantly reduced compared to the corresponding values in the control rats. The Gln level in the hippocampus of the CFSS rats was significantly increased compared to control ($P < 0.05$).

CFSS-induced Histological Changes

Among the hippocampal sub-fields and PFC of the CFSS rats, only the DG showed significantly reduced density of Nissl-positive neurons relative to that in the control rats ($P < 0.05$, Fig. 5C, D), and only the CA3 region showed significantly reduced density of GFAP-IR astrocyte (Fig. 5A, D). The CFSS rats also appeared to have weaker staining for GFAP in the ventral orbital cortex (Fig. 5B).

Discussion

This study was aimed to investigate the cerebral metabolic changes in a depression-like rat model of 14-day CFSS. The main results obtained are summarized as following: (1) the rats subjected to CFSS showed significantly increased immobility time and significantly decreased climbing time in the FST test, indicating development of behavioral

despair in these animals; (2) the rats treated with CFSS showed reduced Glu levels in both the PFC and the hippocampus, similar to what had been observed in clinical MDD patients; (3) the CFSS rats showed decreased density of GFAP-IR astrocytes in the hippocampal CA3 region, weaker staining for GFAP in the ventral orbital cortex and lower density of Nissl-positive neurons in the DG.

The CFSS Model

The rat model of CFSS used in this study is modified from the one established by Kitamura et al. [12]. Stress exposure is an important factor in the development of depressive disorders [16]. The CFSS model simulates an important symptom of clinical depression, behavioral despair, and has been widely used for screening antidepressants [12–15].

In this study, FST and OPT were used to demonstrate the behavioral changes induced by the CFSS treatment. The results showed that, compared to the control rats, the rats subjected to 14-day CFSS exhibited significantly increased immobility time and significantly reduced climbing time in the FST, but did not show any changes in spontaneous exploratory activity by OPT. No significant changes in body weight gain were observed for the CFSS relative to the control rats (data not shown), agreeing with the results of Abel et al. [23]. These data, together, suggest that the CFSS-treated rats in this study had developed a state of behavioral despair [24].

Technical Considerations on Ex vivo ^1H MRS

Measurement of the concentrations of cerebral metabolites by in vivo ^1H MRS is known to be susceptible to errors induced by limited signal-to-noise ratio, motion, partial volume contamination, spectral overlap and contributions to the spectral baseline from broad macromolecule peaks. Because of spectral overlapping, accurate determination of Glu and GABA concentrations with in vivo ^1H MRS often requires special spectral editing techniques. Ex vivo ^1H MRS on brain tissue extract can be performed at higher field strength, thus having higher detection sensitivity and improved dispersion of metabolite peaks. At 500 MHz field

Fig. 3 Representative ^1H NMR spectra of the PFC extracts obtained from a rat in the control (Con) group and a rat in the CFSS group. The inset shows the location (bold lines) where the PFC samples for NMR analysis were taken. Glu: glutamate; GABA: γ -aminobutyric acid; Gln: glutamine; Tau: taurine; NAA: N-acetyl aspartate; Glx: Glu + Gln; Cr: creatine; *m*-Ins: *myo*-inositol; Ace: acetate; Asp: aspartate; Lac: lactate; Ala: alanine

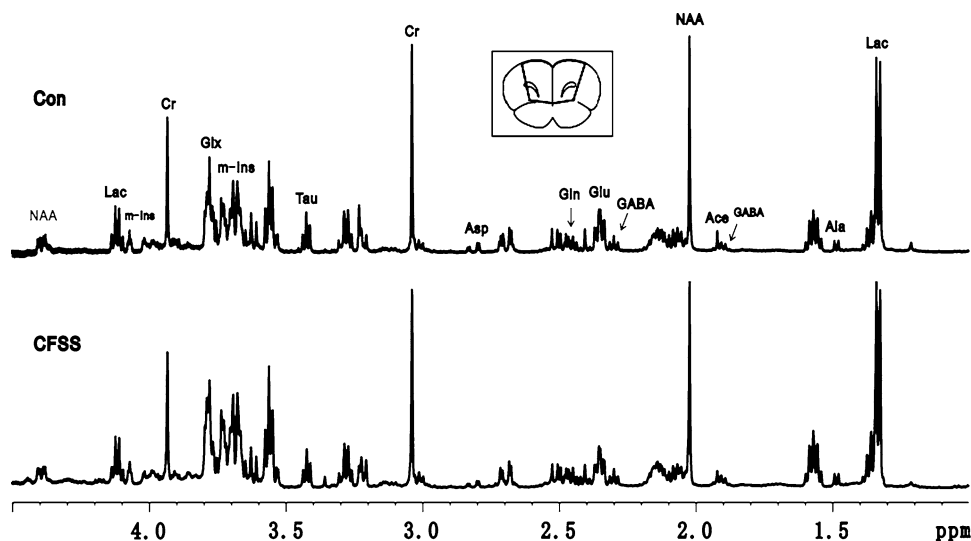


Fig. 4 Metabolite concentrations and Glu/GABA ratios in the PFC (A) and hippocampus (B) of the CFSS rats and control (Con) rats. Glu: glutamate; GABA: γ -aminobutyric acid; Gln: glutamine; Tau: taurine; NAA: N-acetyl aspartate. Glx: Glu + Gln. The results are expressed as mean \pm SD with $n = 6$ for each group, $*P < 0.05$ relative to the control group

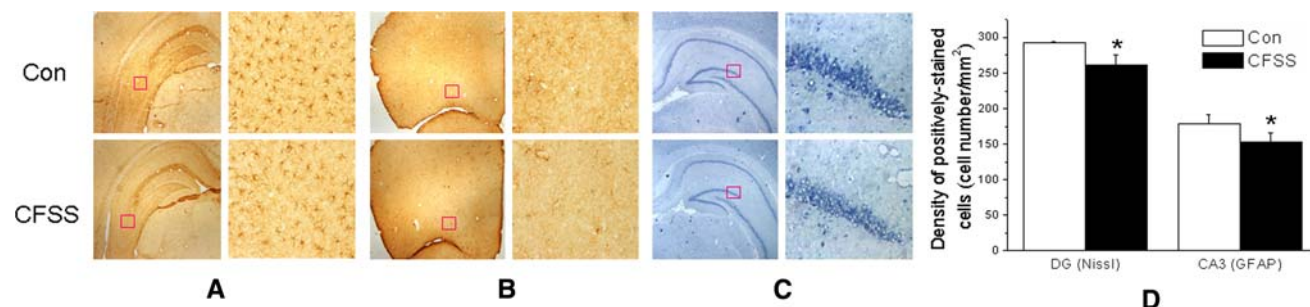
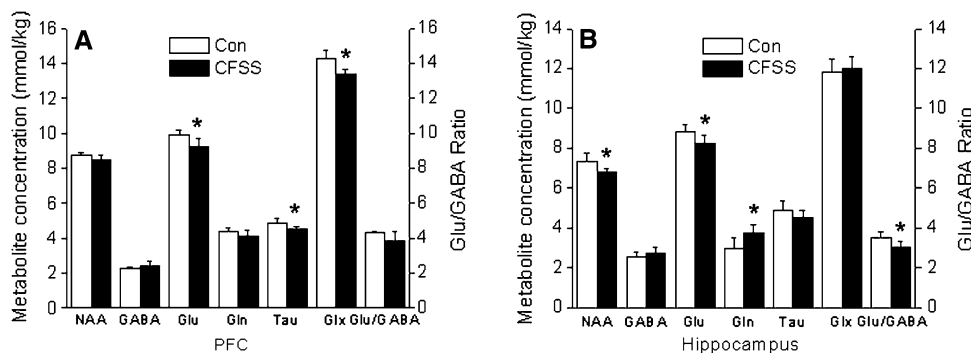


Fig. 5 The results of GFAP immunocytochemistry and Nissl staining. Panels A, B and C show stained brain sections from a control rat (Con, top row) and a CFSS-treated rat (bottom row). The images shown in the left column of each panel were obtained under $40\times$ amplification, while those in the right column were captured under $400\times$ amplification. The open boxes on the images in the left columns indicate the regions magnified in the corresponding right columns.

strength, Glu, Gln and GABA can be detected unequivocally without the need of spectral editing. However, the results of *ex vivo* measurements are known to be susceptible to errors induced by post-mortem metabolism and complicated sample preparation procedures. In this study, the absolute metabolite concentrations in the PFC and hippocampus measured from the control rats were shown to be in

agreement with the previous results obtained in our laboratory using the same sample preparation procedures [20], and those obtained by *in vivo* ^1H MRS on rat brain [21].

To further reduce measurement errors induced by sample preparation procedures, relative metabolite concentrations, referenced to that of total Cr, were reported and compared between the groups. The total Cr level is generally

considered to be relatively constant under a wide range of pathological conditions, and few of the state-of-the-art clinical ^1H MRS studies using absolute quantification have reported a change in total Cr levels in MDD [2].

Changes of Glu, Gln and GABA Levels in CFSS-treated Rats

In this study, the CFSS rats were found to have reduced Glu levels in both the PFC and the hippocampus. The Gln level in the PFC was unaffected by CFSS treatment, and this is in contrast to the case of hippocampus where significantly increased Gln concentration was observed after the 14-day CFSS treatment. Consequently, the Glx level in the PFC of the CFSS rats was found to be significantly reduced compared to control, but not the Glx level in the hippocampus. Previous clinical studies have shown, consistently, that the Glu [8, 9] and Glx [4, 8, 9] levels in the PFC of MDD patients are significantly reduced compared to control. In MDD patients, reduced Glx level was also found in a brain region containing amygdalar gray matter and the anterior one-third of the hippocampus [10]. The differential changes in the Gln level in the hippocampus and PFC probably suggest that the metabolic perturbations involve different metabolic pathways [20]. However, we did not observe any significant changes of GABA levels in the PFC and hippocampus of the CFSS rats. This is different from clinical results showing that the GABA levels in the sub-region of PFC and occipital cortex of MDD patients were significantly reduced relative to control [4, 7]. The reason for this discrepancy is unclear, but could reflect the differences in the pathophysiology underlying CFSS and clinical depression.

It was found in this study that the Glu/GABA ratio was significantly reduced in the hippocampus, but not in the PFC, of the CFSS rats. This is in clear contrast to the results of a recent study showed significantly increased Glu/GABA ratio in both the PFC and hippocampus of depression-like rats bred for learned helplessness (LH) [25]. Human MDD patients appear to have increased Glu/GABA ratio in the occipital cortex [6], but reduced Glx/GABA ratio in the PFC [4]. The differential changes in the Glu/GABA ratio in the LH and CFSS models could be a manifestation of the difference in the pathophysiological mechanisms underlying these two models.

Changes of the Levels of Other Metabolites in CFSS-treated Rats

NAA is a metabolite found predominately in mature neurons, and is thought to be a marker of neuronal integrity and/or viability [26]. The averaged NAA level in

the hippocampus of the CFSS rats was found to be significantly lower than that in the control rats, indicative of possible neuronal injuries in this brain region. This is further confirmed by histology results showing that the density of Nissl-positive neurons in the DG of the CFSS rats was significantly reduced compared to control. It has also been shown that depressed patients and animal model of chronic psychosocial stress treatment have significantly decreased NAA concentration in the hippocampus [27, 28].

The Tau level in the PFC of the CFSS rats was found to be significantly lower than that of the control rats. Tau is known to be involved in inhibitory neurotransmission, neuromodulation, osmotic regulation, membrane stabilization, antioxidation and detoxification [29]. Because Tau is found predominately in glial cells [30], the spectroscopic finding is consistent with the histological results showing that the CFSS showed weaker staining for GFAP in the ventral orbital cortex, relative to control.

PFC and Hippocampus in Depressive Disorders

PFC and hippocampus are probably the two most important brain regions involved in depression and stress. The patients with MDD often show reduced PFC volumes [31], hippocampal volumes and memory deficits compared with the controls [27, 32]. The morphological and functional changes in the PFC and hippocampus of the MDD patients have been attributed to cellular perturbations in these regions. For example, post-mortem studies have reported reductions in the average density and size of neurons and glial cells in the PFC of MDD [33]. It has also been suggested that apoptosis [34], decreased neurogenesis [35] and reduction in neuropil [36] may account for decreased hippocampal volume in the MDD patients detected by neuroimaging.

Many animal experiments have also demonstrated that chronic stress treatment can induce perturbations in the PFC and hippocampus at molecular and cellular levels. In hippocampus, repetitive stress is known to cause lower neurogenesis in DG [37], CA3 dendritic retraction [38], and significantly decreased number and somal volume of astroglia [39]. The rats subjected to repeated restraint stress are known to exhibit PFC dysfunction [40] and dendritic spine loss in the medial PFC [41]. Consistent with the literature data, we found in this study that the CFSS-treated rats have decreased density of GFAP-IR astrocytes in the hippocampal CA3 region, weaker staining for GFAP in the ventral orbital cortex and lower density of Nissl-positive neurons in the DG. Therefore, the metabolic changes found in the PFC and hippocampus of the CFSS rats, at least partially, are manifestations of these pathological changes at cellular level.

Conclusions

In summary, the results of this study suggest that CFSS treatment can induce region-specific changes in the metabolism of excitatory neurotransmitter Glu, and such metabolic alterations, to a large extent, resemble those found in the clinical depression patients. Together, our results indicate that the CFSS model might be useful to study antidepressants specifically targeting the central glutamatergic system.

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