Glial pathology in an animal model of depression: reversal of stress-induced cellular, metabolic and behavioral deficits by the glutamate-modulating drug riluzole

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Abstract

Growing evidence indicates that glia pathology and amino-acid neurotransmitter system abnormalities contribute to the pathophysiology and possibly the pathogenesis of major depressive disorder. This study investigates changes in glial function occurring in the rat prefrontal cortex (PFC) after chronic unpredictable stress (CUS), a rodent model of depression. Furthermore, we analyzed the effects of riluzole, a Food and Drug Administration-approved drug for the treatment of amyotrophic laterosclerosis, known to modulate glutamate release and facilate glutamate uptake, on CUS-induced glial dysfunction and depressive-like behaviors. We provide the first experimental evidence that chronic stress impairs cortical glial function. Animals exposed to CUS and showing behavioral deficits in sucrose preference and active avoidance exhibited significant decreases in ^{13}C-acetate metabolism reflecting glial cell metabolism, and glial fibrillary associated protein (GFAP) mRNA expression in the PFC. The cellular, metabolic and behavioral alterations induced by CUS were reversed and/or blocked by chronic treatment with the glutamate-modulating drug riluzole. The beneficial effects of riluzole on CUS-induced anhedonia and helplessness demonstrate the antidepressant action of riluzole in rodents. Riluzole treatment also reversed CUS-induced reductions in glial metabolism and GFAP mRNA expression. Our results are consistent with recent open-label clinical trials showing the drug's effect in mood and anxiety disorders. This study provides further validation of hypothesis that glial dysfunction and disrupted amino-acid neurotransmission contribute to the pathophysiology of depression and that modulation of glutamate metabolism, uptake and/or release represent viable targets for antidepressant drug development.

Keywords

antidepressant; stress; glia; glutamate; glutamate transporter; metabolism

Introduction

Major depressive disorder (MDD) is a common and disabling illness affecting a rising percentage of the world's population. At present, nearly all of the medications available for the treatment of MDD have been developed out of the monoaminergic deficit hypothesis of depression that arose in the mid 1960s.[1] Although this approach has led to great advances in our ability to treat MDD, the limitations of our current armamentarium of antidepressant drugs are becoming increasingly evident.[2] This realization presents a strong impetus to search beyond the monoaminergic systems for a better understanding of the pathophysiological mechanisms underlying MDD, and to consider these newly identified mechanisms in the search for novel antidepressant medications.

Growing evidence indicates that glial elements are involved in the neuropathology of several neuropsychiatric illnesses including MDD.[3] Post-mortem studies of tissues from patients with MDD describe a reduced number and an altered morphology of glial cells in several brain regions, in particular the prefrontal cortex (PFC).[3–7] Other post-mortem studies demonstrating altered expression of glial fibrillary associated protein (GFAP), glial-specific excitatory amino-acid transporters (EAATs) and glutamine synthetase (GS) in tissue from individuals with MDD[8,9] suggest that the glial cell abnormalities include changes in astrocytic cell function. Recent studies provide evidence that stress exposure may be related to some of the reported glial cell pathology by demonstrating that animals exposed to chronic stress have a decreased glial density in the hippocampus[10] and a reduced production of glial cells in the adult hippocampus and PFC.[11–13]

Stress-related effects on glutamate (Glu) neuro-transmission are increasingly implicated in the pathophysiology and pathogenesis of MDD,[3,14–16] and there is now strong evidence that marked abnormalities exist within the glutamatergic and GABAergic amino-acid neurotransmitter (AANt) systems of individuals with MDD.[17] In this context, the role of glial cells in the regulation of the AANt system function[18,19] through the clearance of Glu from the extracellular space via the EAATs[20] and recycling of Glu through the glutamate/glutamine (Glu/Gln) cycle[21] seems especially relevant to the pathophysiological processes underlying mood disorders.[17] Specifically, impaired glial cell function would be predicted to lead to increased glutamatergic activation, especially at extrasynaptic sites that have been shown to promote neurotoxic-like effects.[22,23] Recent evidences showing NMDA antagonists to produce rapid antidepressant responses have further fueled interest in this model.[24] The hypothesis that increased levels of extracellular Glu could be related to depressive-like behaviors, and that increased Glu uptake could have protective, antistress or antidepressant-like effects is supported by a recent study demonstrating that ceftriaxone, a β-lactam agent known to increase EAAT expression,[25] has significant antidepressant-like properties in the tail suspension and forced swim tests (FSTs).[26]

Riluzole, a drug currently used to slow the progression of amyotrophic lateral sclerosis, is believed to alter Glu neurotransmission by both decreasing presynaptic release and facilitating glial cell Glu uptake.[27–31] Interestingly, riluzole has also been shown to be effective in open-label studies in several psychiatric disorders including treatment-resistant MDD.[31] The goal of the current study is to test two related hypotheses: (1) chronic stress leads to impaired astrocytic function and reduced AANt cycling, resulting in AANt perturbations that are associated with characteristic stress-induced behaviors, and (2)

riluzole, a drug that facilitates Glu uptake will attenuate the effects of stress on AANt cycling, and reverse or attenuate the effects of stress on tests of anhedonia and despair.

Materials and methods

Animals

Male Sprague–Dawley rats (Charles River) were housed in wire bottom cages under a 12-h light/12-h dark cycle at constant temperature (25 °C) and humidity with free access to food and water except when animals were subjected to light disturbance or deprivation stressors during the chronic unpredictable stress (CUS) procedure. Animals weighed between 250 and 300 g at the time of the first stressor. Experiments began after at least 1 week of habituation to the housing conditions. Animal use procedures were in accordance with the Yale University Care and Use of laboratory animals guidelines.

Chronic unpredictable stress procedure

CUS is an experimental procedure in which animals are exposed to a variable sequence of mild and unpredictable stressors. Our CUS procedure was successfully used in the laboratory to produce behavioral changes as well as alteration of glial cell proliferation in the PFC.[12] The CUS animals were subjected to exactly the same sequence of 12 stressors (2 per day for 35 days) described in Banasr *et al.*[12]: cage rotation, light on, light off, cold stress, isolation, crowding, cold swim stress, food and water deprivation, wet bedding, stroboscope, cage tilt and odor exposure. To simulate a realistic course of antidepressant intervention, animals were first exposed to CUS for 15 days, and then were administered with riluzole (4 mg/kg, i.p) daily for 21 days with continued CUS procedure. The dose used in this study was similar to Risterucci *et al.*[32] and was shown to block behavioral deficit induced by PFC ibotenate-induced excitotoxic lesion. On days 15 and 35 of the study, animals were tested in the sucrose preference test (SPT). On day 28, animals were tested in the active avoidance test (AAT). All animals were tested for locomotor activity on days 15, 28 and 35.

Sucrose preference test

The SPT was performed on days 15 and 35 and consisted in 48 h exposition to a palatable sucrose solution (1%; Sigma, St Louis, MO, USA), followed by 4 h of water deprivation and a 1-h exposure to two identical bottles, one filled with sucrose solution and the other with water. This procedure was adapted from Willner[33] and has been previously used successfully in our lab.[12] Sucrose and water consumption was determined by measuring the change in volume of fluid consumed. Sucrose preference was defined as the ratio of the volume of sucrose vs water consumed during the 1-h test.

Active avoidance test

The active avoidance procedure was performed in one learned helplessness chamber of custom-built shuttle boxes (Med Associates). After a 5 min habituation, animals received 30 randomized escapable footshocks at an intensity of 0.65 mA. In each shock trial, the gate separating the two halves of the shuttle box opened 5 s before shock onset and remained open for the duration of the shock. The average intertrial interval was 60 s (range 20–100 s). The first 5 trials required one crossing to terminate footshock (FR1) and the remaining 25 trials required two crossings (FR-2). Results are expressed as number of escape failures, that is, the number of times that the animal did not terminate the footshock.

In situ *hybridization*

After all animals were tested in behavior, a subset was killed by decapitation 24 h after the last stressor and/ or the last drug injection for in *situ* hybridization and the other subset was

used for the nuclear magnetic resonance (NMR) study. Brains were removed, rapidly frozen on dry ice and stored at 80 °C. Cryocut sections (14 μm thickness) containing prelimbic cortex (4.2–2.2 mm from bregma) were subjected to in *situ* hybridization analysis as previously described.[34] Briefly, radiolabeled (^{35}S) riboprobes were generated by in *vitro* transcription (Megashortscript; Ambion) from PCR product amplified from whole brain using gene-specific primers. The GFAP, GLT-1, GLAST, GS probes were verified by sequencing and previously used in our laboratory.[34] The optical density was quantified within a 500 μm^2 box in the prelimbic cortex using NIH ImageJ software. Results are expressed as mean±s.e.m. percentage from control (home cage animals injected with saline for 21 days).

[2-^{13}C]acetate infusion and sample preparation

To determine whether CUS and/or chronic riluzole treatment induce changes in glial metabolism, we used a similar approach to Chowdhury *et al.*[35] based on infusion of [2-^{13}C]acetate followed by ^{1}H-[^{13}C]-NMR *ex vivo*. At 24 h after the last stressor or/and injection, rats were anesthetized with urethane (1.5 g kg^{-1}, i.p.) and 30 min later, [2-^{13}C]acetate (99 atom%; Cambridge Isotopes, 2 M, pH 7) was infused through a tail vein. The solution was delivered by variable rate infusion (from 0–15 s at a rate of 3.12 ml min^{-1} kg^{-1} followed by 436 ml min^{-1} kg^{-1} from 15 s to 4 min, at 249 μl min^{-1} kg^{-1} from 4 to 8 min and 125 μl min^{-1} kg^{-1} from 8 to 15 min). Immediately after infusion, rats were killed using a directed microwave pulse (5 kW, Model TMW 6402C; Muromachi Microwave Fixation System) to the head to quickly arrest metabolism allowing brain tissue to be removed without post-mortem changes. PFC was then dissected, frozen in N_2 liquid and stored at −80 °C until concentrations of the major amino acids (Glu, GABA and Gln) in these tissue samples as well as their ^{13}C enrichments were determined using ^{1}H-[^{13}C]-NMR spectroscopy. Blood was sampled from the heart at the end of experiment for determination of the isotopic fractional enrichments and concentrations of the infused substrate. After centrifugation, collected blood was frozen in liquid N_2, and stored at −80 °C for subsequent analysis.

PFC metabolites were extracted from frozen tissue (130–150 mg) as described previously.[36] Briefly, tissue was ground with 0.1 mol l^{-1} HCl in methanol (1:2, wt/vol). A known quantity of [2-^{13}C] glycine (0.5 μmol) was added as an internal concentration reference. The tissue powder was homogenized with ice-cold ethanol (1:6, wt/vol; 60% ethanol) then clarified by centrifugation (20 000 g). The supernatant was lyophilized and resuspended in 500μl of a phosphate-buffered (100 mmol l^{-1}, pH 7) deuterium oxide (Cambridge Isotope Laboratories) solution containing 0.5 mmol l^{-1} 3-trimethylsilyl[2,2,3,3-D4]-propionate (TSP), which served as a chemical shift reference.

Frozen blood plasma samples were thawed for direct measurement of glucose (Beckman Glucose Analyzer II, Model 6517; Beckman Instruments Inc.). The remaining plasma was mixed with deuterium oxide and passed through a centrifugal filter (10 kDa cutoff, Nanosep, Centrifugal Devices; Pall Life Science). Samples were loaded into 5 mm NMR tubes along with known quantities of TSP and formate, the latter serving as a concentration standard.

Nuclear magnetic resonance analysis of cortical extracts

Fully relaxed ^{1}H-[^{13}C]-NMR spectra of plasma and cortical extracts were acquired at 11.7 T (^{1}H resonance frequency of 500.13 MHz) using a Bruker AVANCE spectrometer (Bruker Instruments). ^{1}H-[^{13}C]-NMR spectra were acquired as two subspectra—one involving broadband ^{13}C inversion pulses applied in alternate scan blocks, whereas ^{13}C-decoupling was applied in both. Subtraction of the scans obtained with ^{13}C inversion (^{12}C–^{13}C) from those without inversion (^{12}C+^{13}C) gave the difference spectrum (2×^{13}C), containing

only ^{13}C-coupled ^{1}H resonances at twice the true intensity. The total carbon isotope composition was given by the ^{12}C+^{13}C subspectrum. The ^{13}C atom percentage enrichment was calculated as the ratio, ^{13}C/(^{12}C+^{13}C)×100, followed by subtraction of 1.1% to remove ^{13}C arising from natural abundance.[35] Absolute concentrations of metabolites were determined relative to [2-^{13}C]glycine, added during tissue extraction as an internal concentration reference. The isotopic ^{13}C enrichments of Glu-C4, C3, GABA-C2, Gln-C4, succinate (Suc)-C2+C3, aspartate (Asp)-C3, alanine (Ala)-C3 and lactate (Lac)-C3 were calculated from the ratio of the areas of these resonances in the ^{1}H-[^{13}C]-NMR difference spectrum (2×^{13}C only) and the nonedited spectrum (^{12}C+^{13}C).

Nuclear magnetic resonance analysis of blood samples

The concentration and ^{13}C isotopic enrichment of plasma [2-^{13}C]acetate (2-CH$_3$, 1.9 p.p.m.) and total acetate concentration were determined using ^{1}H-NMR without ^{13}C decoupling using a repetition time of 20 s. Formate was added to the sample as a concentration standard. The isotopic ^{13}C enrichment of acetate-C2 (1.9 p.p.m.) was calculated by dividing the areas of the ^{13}C satellites with the total area (^{12}C+^{13}C) of their respective resonances.[36]

Statistical analysis

Statistical differences were determined by analysis of the variance (ANOVA, StatView 5) followed by Newman–Keuls *post hoc* analysis. Repeated-measures ANOVAs were used to analyze the effect of the treatments when the same behavioral test was used at different time points for the same individual. This analysis was followed by Dunnett's *post hoc* analysis. The F-values and group and experimental degrees of freedom are included in the legends of the figures. For experiments with two groups, Student's *t*-test was used. The level of statistical significance was set at $P < 0.05$ using two-tailed tests.

Results

CUS-induced effects on depressive-like behaviors are reversed by chronic riluzole treatment

In this study, we used a well-documented animal model of depression, CUS that results in multiple behavioral effects, many of which are relevant to those observed clinically, most notably anhedonia.[33] Moreover, these changes are reversed after chronic, but not acute antidepressant treatment, a time-dependent effect that is poorly modeled by other behavioral paradigms (for example, learned helplessness or FST). The CUS procedure used in this study was the same procedure reported previously by our lab and consists of 12 different randomized stressors, 2 per day for 35 days.[12] Home cage control (CTR) and CUS animals were subjected to the SPT, which is used as an index of the hedonic-like state of animals on days 15 and 35 (Figure 1; experimental design).

We found that CUS induces a significant decrease in the ratio of sucrose vs water consumed after 15 days of stress compared to CTR animals (Figure 2a; $n = 16$ per group; *t*-test, $P < 0.05$). No difference between groups was observed in total fluid consumption for the 1 h test. In addition, no changes in spontaneous locomotor activity were found at this time point (Supplementary Table S1). To simulate a realistic course of antidepressant intervention, animals were then split into two experimental groups (based on their SPT performances, $n = 8$ per group) and injected with riluzole (4 mg kg^{-1}) or saline i.p. daily for 21 days with continued CUS procedure (Figure 1; experimental design). Animals were tested in the AAT, index of helplessness or despair,[37] on day 28 and retested in SPT on day 35.

CUS animals injected with saline (CUS+SAL) showed a higher number of escape failures than CTR animals injected with saline (CTR+SAL) (Figure 3; $n = 8$ per group; two-way

ANOVA, *post hoc* Student–Newman–Keuls, $P < 0.01$). Although behavioral performances in AAT of CTR animals injected with riluzole (CTR+RIL) and CUS animals treated with riluzole (CUS+RIL) were not different from that of CTR+SAL group, the number of escape failures was significantly lower in CUS+RIL group compared to CUS+SAL group (Figure 3a; two-way ANOVA, *post hoc* Student–Newman–Keuls, $P < 0.001$). Latency to escape was also increased in CUS+SAL animals compared with the other groups. This effect was significant from the second FR-2 block of five escapable shocks (footshocks 10–15) to the end of the test. CUS+RIL animals showed performances in AAT significantly different from CUS+SAL as soon as the first FR-2 block of five escapable shocks (Figure 3b; two-way repeated-measures ANOVA, *post hoc* Dunnett's, $P < 0.01$). These results demonstrate that CUS induces helplessness in the AAT and that riluzole prevents this effect.

On day 35, all animals were subjected to a second SPT. We confirmed that 35 days of CUS induce decreases in the ratio of sucrose to water consumed when compared with CTR +SAL12 (Figure 2b; $n = 8$ per group, two-way ANOVA, *post hoc* Student–Newman–Keuls, $P < 0.01$). Although the effect of CUS on sucrose preference was seen as early 15 days, continuation of CUS for 21 more days significantly increases the magnitude of this effect (Figures 2a and b; repeated-measures ANOVA, *post hoc* Dunnett's, $P < 0.05$). Finally, we demonstrate that chronic treatment with riluzole significantly reversed the effect of CUS on sucrose preference (Figure 2b; twoway ANOVA, post hoc Student–Newman–Keuls, $P < 0.01$). Again, total fluid consumption during the 1-h test did not differ between groups. In addition, changes in spontaneous locomotor activity were analyzed on days 28 and 35 in the same animals and did not differ between groups throughout the experiment, demonstrating that the results obtained in AAT and SPT were independent of this experiment bias (Supplementary Table S1). In summary, our results demonstrate that CUS induces anhedonia and helplessness and that these behavioral deficits can be prevented and/or reversed by chronic treatment with riluzole.

We also characterized the antidepressant-like properties of acute and chronic (10 days) treatment with riluzole in the FST, a behavioral test used as a screen to predict antidepressant-like properties of new compounds (Supplementary Figures S1a and b). Although time spent immobile was decreased in both conditions, only chronic treatment with riluzole induced a significant effect (Supplementary Figure S1b; $n = 6$–7 per group; *t*-test, $P < 0.05$). We also analyzed the effects of acute and chronic treatment with riluzole on spontaneous locomotor activity (Supplementary Table S1). Whereas chronic riluzole has no effect, acute riluzole induces a statistically significant decrease in locomotor activity (Supplementary Table S1; $n = 6$ per group; *t*-test, $P < 0.05$). Thus, it is not possible to rule out the fact that an acute antidepressant-like effect of riluzole in the FST was masked by a general decrease in locomotor behavior. In any case, these results show that chronic treatment with riluzole has antidepressant-like properties in CUS-induced anhedonia and helplessness, as well as the FST.

CUS effects on Glu/Gln cycling and GABA synthesis are reversed by chronic riluzole treatment

To determine whether chronic stress or riluzole treatment alters glial metabolism and AANt cycling and/or synthesis, we analyzed the changes in concentrations of amino acids and metabolites occurring in the PFC of CUS animals and/or riluzole-treated animals. For this study, we used a similar approach to Chowdhury et al.[35] based on infusion of [2-^{13}C]acetate, which has been shown to be metabolized preferentially in astroglia[38] (see Figure 4a), followed by ^{1}H-[^{13}C]-MRS *ex vivo*. The initial results demonstrate that average concentrations of acetate were similar in plasma samples from all groups (mean±s.e.m.; CTR+SAL = 16.1±1.0 mmoll^{-1}; RIL = 17.3±0.5 mmoll^{-1}; CUS+SAL = 15.9±1.0 mmoll^{-1} and CUS+RIL = 15.8±0.8 mmoll^{-1}, $n = 8$ per group). Similar results were found for the

percentage ^{13}C enrichments: mean±s.e.m.; CTR+SAL = 91.2±0.5%; CTR+RIL = 92.3±0.8%; CUS+SAL = 92.8±0.4% and CUS+RIL = 93.0±0.7%). In addition, total levels of amino acids and metabolites were determined in the different groups by averaging the respective [2-^{13}C]acetate infusion data; total PFC concentrations of Glu, GABA, Gln, Asp, Suc, Ala, NAA, Cre and Myo-Ins were similar in all animal groups (not shown). These results indicate that changes in PFC levels of the ^{13}C-labeled amino acids found in this study are not related to general metabolic alterations or changes in total amino-acid concentrations. We did not detect β-hydroxybutyrate[35] in the blood or brain extract spectra of any group, thus indicating the treatments did not induce ketosis in the animals.

Concentrations of ^{13}C-labeled amino acids from [2-^{13}C]acetate were found to be altered in PFC samples from CUS- and riluzole-treated animals (Figures 4b–d; Table 1). Indeed, whereas ^{13}C concentrations of Glu-C4, Gln-C4 and GABA-C2 were similar in both CTR +RIL and CTR+SAL groups, the CUS+SAL group showed significant reductions of ^{13}C concentrations of Glu-C4 (Figure 4b; $n = 8$ per group; two-way ANOVA; *post hoc* Student–Newman–Keuls, $P < 0.05$), Gln-C4 (Figure 4c; $P < 0.01$) and GABA-C2 (Figure 4d; $P < 0.05$) when compared to CTR+SAL. We found similar reductions in the percentage of ^{13}C enrichments of Glu-C4, and GABA-C2 when comparing CTR+SAL vs CUS+SAL groups (Table 1). These results suggest that CUS decreases glial metabolism and AANt cycling in the rat PFC.

Interestingly, we also found that ^{13}C concentrations of Glu-C4 and GABA-C2 in CUS+RIL animals were significantly different from CUS+SAL animals (Figures 4b–d; two-way ANOVA, *post hoc* Student–Newman–Keuls, $P < 0.05$). Although riluzole's effect on Gln-C4 ^{13}C concentration in CUS+RIL animals did not reach significance when compared to CUS+SAL, a strong trend was observed, and Glu-C4 ^{13}C concentrations were not significantly different from CTR+SAL or CTR+RIL group (Figures 4b–d; two-way ANOVA, *post hoc* Student–Newman–Keuls, $P = 0.073$). In addition, chronic riluzole treatment significantly increased the percentage ^{13}C enrichments of all three metabolites in CUS-treated animals (Table 1). This suggests that riluzole reverses the effects of CUS on glial metabolism and AANt cycling.

CUS and riluzole affect expression of glial-specific markers

To determine whether glial alterations are present in our animal model of depression, we investigated the effects of CUS and/or riluzole on expression levels of genes specific to glial cells using in situ hybridization. We analyzed the changes in expression levels of GFAP, the two glial-specific EAATs (GLT-1/EAAT$_2$ and GLAST/ EAAT$_1$) and GS. We found that CUS induces a significant 20% decrease in GFAP mRNA expression and no change in expression level of the other markers (Figures 5a–c; CTR+SAL: n=4, CUS+SAL: n = 5; two-way ANOVA, *post hoc* Student–Newman–Keuls, $P < 0.05$). Although chronic riluzole had no effect on GFAP expression in nonstressed animals, this treatment reversed the effect of CUS (Figures 5a and b; RIL = 4, CUS+SAL: $n = 5$, CUS+RIL: $n = 5$; two-way ANOVA, *post hoc* Student–Newman–Keuls, $P < 0.05$). This decrease in GFAP expression suggests that the morphology and/or architecture of astrocytes may be altered by CUS and that riluzole treatment blocks these changes.

In addition, we found that CUS has no effect on the expression of GLT-1 (Figures 5d and e), GLAST or GS (Table 2). However, chronic riluzole treatment induced a significant ~30% increase in GLT-1 expression in the PFC in the CUS+RIL group (Figures 5d–f; two-way ANOVA, *post hoc* Student–Newman–Keuls, $P < 0.05$). No change was observed on the expression of GLAST and GS transcripts following riluzole treatment in stressed and nonstressed animals (Table 2). Increased GLT-1 expression found in this experiment

suggests that riluzole treatment may attenuate glutamatergic hyperactivity, classically described after stress.[39]

Discussion

This study provides the first experimental evidence of impaired cortical glial function in an animal model of depression. The results demonstrate that animals exposed to CUS exhibit behavioral deficits in tests measuring helplessness and anhedonia, as well as decreases in ^{13}C-acetate metabolism and GFAP mRNA expression in the PFC. The cellular and behavioral alterations induced by CUS were reversed or blocked by chronic treatment with the Glu-modulating drug riluzole. The beneficial effects of riluzole on CUS-induced anhedonia and helplessness clearly define chronic riluzole as an antidepressant treatment in this model. Chronic riluzole was also able to reverse the CUS-induced decrease in ^{13}C-acetate metabolism and GFAP expression. These effects were associated with a selective increase in the expression of the glial-specific EAAT, GLT-1. Together, our results suggest that chronic stress alters glial cell function and that the cellular mechanism of riluzole antidepressant action may involve the attenuation of glial dysfunction and an enhancement of extrasynaptic Glu clearance.

Glial impairment in an animal model of depression

Stress-based animal models are a leading approach for analyzing cellular and molecular mechanisms underlying the pathophysiology of depression. These models have also been of tremendous utility in testing antidepressant properties of new compounds. CUS is a dependable depression model with high face, predictive and construct validity.[33] CUS simulates realistic conditions for human depression and results in multiple behavioral changes similar to those observed clinically. In this study, we confirmed that our CUS procedure decreases sucrose preference,[12] a measure of anhedonia in rodents[33] and we show that the same procedure induces helplessness, demonstrated by increased escape failures in AAT. Knowing that anhedonia and helplessness are major symptoms of human depression (Diagnostic and Statistical Manual of Mental Disorders, 4th edn), these results provide further evidence of the validity of CUS and support the hypothesis that cellular changes found in this model may also occur in patients affected by stress-related illnesses.

At the cellular level, we found that animals subjected to CUS show decreased GFAP mRNA expression in the PFC. Decreased GFAP immunostaining and a reduced number of GFAP-positive cells were recently reported after chronic psychosocial stress in the hippocampus, another major limbic brain region involved in depression.[10] As two previous studies,[11,12] failed to demonstrate any effects of stress on the proliferation of GFAP expressing cells, it does not appear the findings are a result of decreased astrocyte proliferation, but rather an effect on mature cortical astrocytes. Although hippocampal glial loss was not found in post-mortem tissue from depressed patients,[40] studies have consistently reported a decreased number and altered morphology of glial cells in several regions of the frontal cortex.[3] This glial pathology has been associated with decreased GFAP immunostaining.[41] As GFAP is a key cytoskeletal protein of astrocytes, this could be related to observed morphological alteration of cortical astrocytes. Whether the glial morphological changes found in the human brain or in this study are associated with alteration of glial function or behavioral deficits remain unknown.

Abnormal increases in Glu efflux have been reported in the PFC in response to stress and, corticosterone treatment,[39] and elevated levels of Glu have been recently found in PFC tissue of depressed subjects.[42] This increase in Glu is postulated to contribute to the morphological alterations observed in limbic regions in MDD and after stress.[16] In addition to the important role of astrocytes in nurturing neurons, they are actively involved in

maintaining synaptic transmission.[43] Glu clearance is believed to be a major function of EAATs located on glial cells. Although we did not find altered expression of GLAST or GLT-1 mRNA in CUS animals, we cannot rule out the possibility of decreased protein levels or transporter activity. Indeed, there are reports suggesting loss of GFAP can decrease EAAT activity through alterations of GLT-1 trafficking at the membrane in the cortex and hippocampus.[44] Alterations of EAAT activity and glial metabolism are consistent with the results obtained in our ^{13}C-acetate analysis. Because glial cells are the main cell type responsible for uptake and metabolism of ^{13}C-acetate in the brain,[38] the lower ^{13}C labeling of glial (Gln-C4) and neuronal (Glu-C4 and GABA-C2) products of AANt cycling in PFC extracts indicates that glial metabolism is decreased in animals subjected to CUS. The AANt cycling is also dependent on GS, a glial-specific enzyme responsible for the recycling of neuronally released Glu and GABA into glutamine.[21,45] Even though we did not see changes in GLT-1, GLAST or GS mRNA expression after CUS, the results obtained in the ^{13}C-acetate indicate that Glu flux through these enzyme/transporters is reduced in astrocytes. In support of this, a previous study has shown that glial-mediated Glu uptake is reduced after chronic stress in rodents.[46] In addition, recent work carried out in our lab demonstrated that local infusion of a gliotoxin in the PFC resulting in decreases in GLT-1, GS and the number of astrocytes was sufficient to induce a depressive-like phenotype similar to CUS (Banasr and Duman 2007, 37th Annual Meeting of the Society for Neurosciences, 383.2). Together, our results along with data from the literature suggest that dysregulation of the primary functions of astrocytes in the PFC is associated with stress and depression and may contribute to the expression of the depressive-like symptoms.

Riluzole: an antidepressant that acts on glia

In this study we demonstrate that the cellular and behavioral alterations induced by CUS are reversed and/or blocked by chronic treatment with riluzole. The antidepressant actions of riluzole were only evident after chronic treatment, including in the FST, which is typically responsive to acute antidepressants. Although acute riluzole induced a trend in decreasing time spent immobile in the FST this effect was not significant and possibly due to the sedative properties of riluzole.[31] This sedative-like effect was confirmed in a parallel group in which acute riluzole decreased locomotor activity, however the locomotor suppressant effects of riluzole were not seen with chronic treatment. In CUS animals, chronic riluzole treatment reversed helplessness in the AAT and anhedonia in SPT. The efficacy of riluzole in these three behavioral tests clearly defines riluzole as an antidepressant and further validates the findings of several open-label clinical studies demonstrating riluzole's efficacy in the treatment of mood and anxiety disorders.[31]

At a cellular level, chronic riluzole treatment increased GLT-1 mRNA expression in the PFC. The increased GLT-1 expression may provide a mechanism contributing to the increased Glu uptake that is observed following riluzole treatment.[27–30] Through the enhanced uptake of riluzole via EAATs it is possible that riluzole helps to prevent the over-stimulation of extrasynaptic Glu receptors, a process believed to result in an excitotoxic cascade leading to a variety of pathological consequences.[23] Chronic riluzole treatment also prevented or reversed the CUS-induced decrease in GFAP expression suggesting that the drug may reverse or block the glial morphological changes (atrophy or loss) found in the PFC of individuals with MDD. This effect would be consistent with riluzole's reported protective effects on glial cells against Glu toxicity.[47] These results support the hypothesis that enhancement of Glu uptake is a new cellular mechanism for antidepressant action. This is consistent with another recent study in which ceftriaxone, which also increases Glu transport,[25] was reported to have antidepressant properties in FST, tail suspension and novelty suppressed feeding.[26]

Finally, chronic riluzole also blocks the CUS-induced decrease in glial metabolism as the reductions in ^{13}C labeling Glu-C4, Gln-C4 and GABA-C2 found after chronic stress were reversed by riluzole. The beneficial effect of riluzole on glial metabolism and glial function supports the hypothesis that a glioprotective drug may have antidepressant properties, and that glial dysfunction in the PFC contributes to the expression of the symptoms of MDD.[13,48] It is important to note that glioprotective properties have also been reported with typical antidepressant agents. Fluoxetine treatment prevented glial loss in the hippocampus of rats subjected to chronic psychosocial stress,[10] and reversed the decrease in glial cell proliferation observed in the PFC after chronic stress.[11,12] Preliminary data from our laboratory (data not shown) also suggest that chronic fluoxetine may increase GLT-1 mRNA expression in CUS-treated animals.

These glial changes are likely associated with changes in neuronal function in the PFC. Many studies show alterations in the morphology of PFC neurons after stress[49] similar to those observed in post-mortem tissue of depressed patients.[3] Questions still remain regarding whether these neuronal changes are the primary cause or a consequence, or occur in parallel to the glial dysfunction.[50] However, the highly consistent finding of glial loss and atrophy even in younger individuals with MDD suggests that glial dysfunction may precede neuronal atrophy.[3] If so, then antidepressants that protect against glial loss, insure glial function and normalize Glu and GABA metabolism and neurotransmission will be of tremendous help in the development of antidepressants with a new mechanism of action.

In conclusion, this series of studies demonstrate that CUS causes changes in glial cell AANt metabolism, the expression of GFAP the major intermediate filament protein in mature astrocytes, and produces a depressive-like phenotype. These findings are consistent with a growing number of studies showing significant glial cell pathology and markedly abnormal AANt concentrations in individuals with MDD. Furthermore, we show that riluzole, a drug known to modulate Glu neurotransmission and facilitate glial Glu clearance, reverses several of the cellular, physiological and behavioral consequences of CUS. This result is consistent with evidence that riluzole and other Glu-modulating agents are efficacious in treating severe treatment-resistant MDD. In sum, although these findings do not conclusively identify a patho-physiological mechanism relating stress, glial pathology and behavior, they do support the general hypothesis that glial cell pathology and AANt abnormalities contribute the pathophysiology of MDD, and that this pathological process can be targeted in the development of novel antidepressant medications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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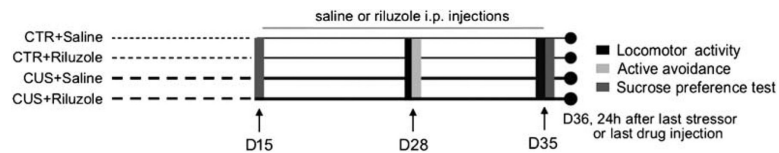
Figure 1.
Timeline of experimental procedures. Rats are handled daily (home cage control, CTR) or subjected to the chronic unpredictable stress (CUS) procedure for 35 days. Animals are administered saline or riluzole (4 mg/kg) for the 21 last days of the experiment. The efficacy of CUS or riluzole on behavioral performances of the animals in locomotor activity, active avoidance and sucrose preferences test are measured.
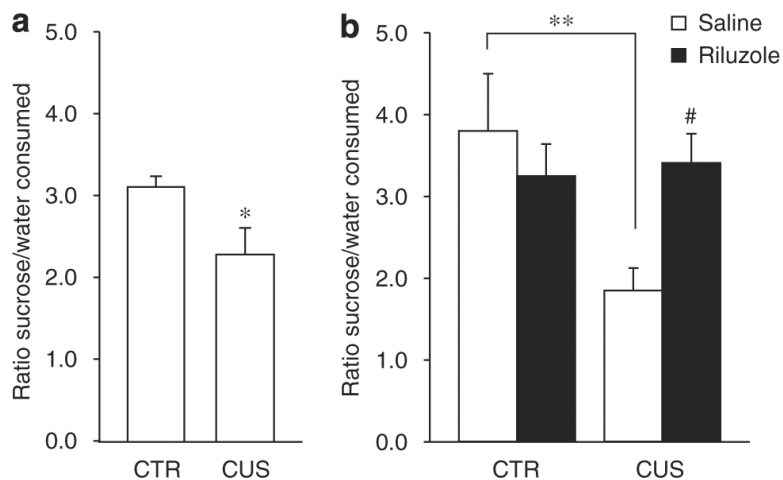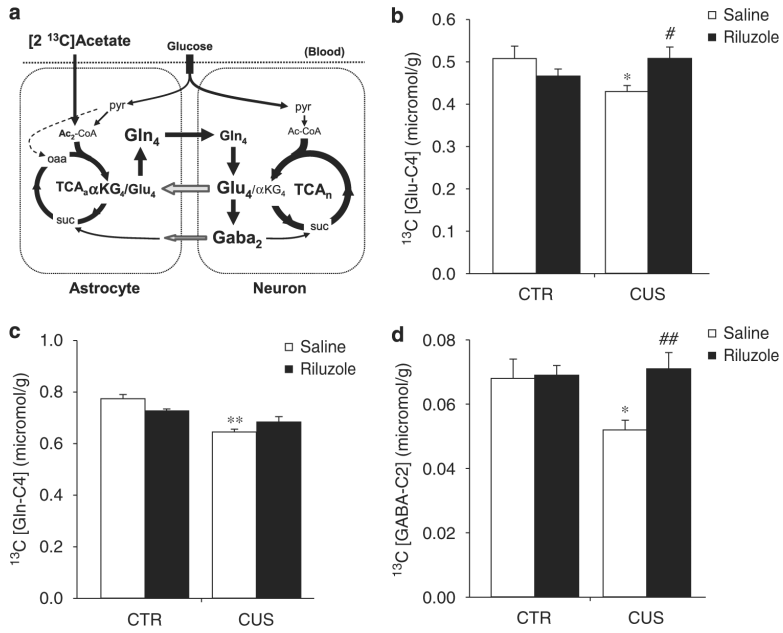
Figure 2.
Effects of chronic unpredictable stress (CUS) and riluzole in the sucrose preference test. (**a**) On day 15, CUS animals showed a significant decrease in sucrose preference when compared to home cage control (CTR) animals ($t_{30} = 2.36$, $P = 0.025$). (**b**) Sucrose preference was decreased by 35 days CUS exposure ($F_{1,28} = 3.42$, $P < 0.01$) and was reversed by chronic riluzole treatment ($F_{1,28} = 5.258$, $P < 0.05$). Error bars represent s.e.m. *$P < 0.05$, **$P < 0.01$ compared to CTR and #$P < 0.05$ compared to CUS, two-way analysis of the variance (ANOVA), Student–Newman–Keuls *post hoc* analysis.
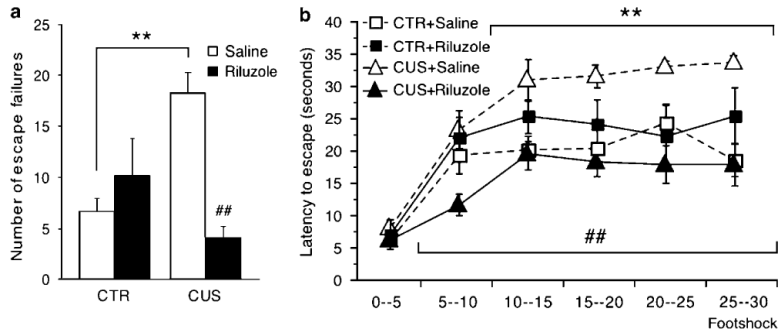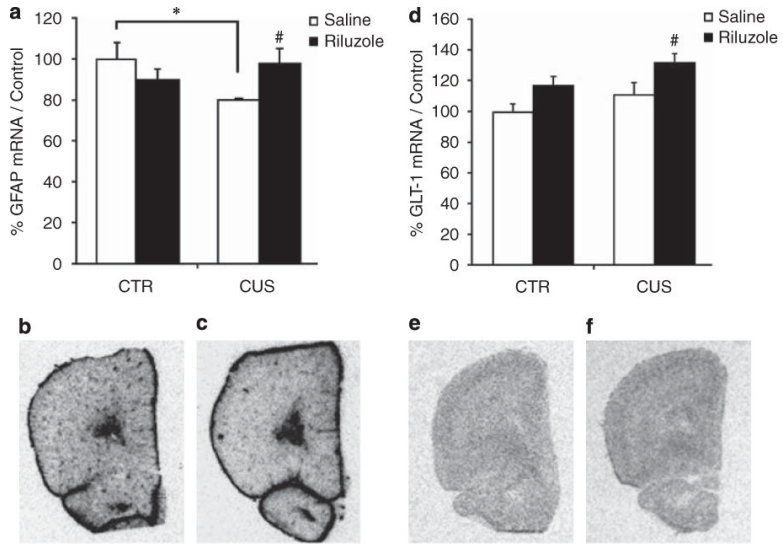
Figure 3.
Effects of chronic unpredictable stress (CUS) and riluzole in the active avoidance test. (**a**) CUS animals showed a significant increase of escape failures ($F_{1,28} = 7.798$, $P < 0.01$) and this effect was reversed by chronic riluzole treatment ($F_{1,28} = 19.05$, $P < 0.001$). Error bars represent s.e.m. **$P < 0.01$ compared to home cage control (CTR) and ##$P < 0.01$ compared to CUS, two-way analysis of the variance (ANOVA) and Student–Newman–Keuls post hoc analysis. (**b**) Latency to escape was significantly increased in CUS animals ($F_{1,28} = 7.79$, $P < 0.01$) starting from the second FR-2 block of five escapable shocks (footshocks 10–15) to the end of the test ($F_{1,168} = 27.37$, $P < 0.01$). Riluzole-treated animals showed performances in this test significantly different from CUS ($F_{1,28} = 16.45$, $P < 0.01$) as soon as the first FR-2 block of five escapable shocks ($F_{1,168} = 45.57$, $P < 0.01$). Error bars represent s.e.m. **$P < 0.01$ compared to CTR and ##$P < 0.01$ compared to CUS, Two-way repeated-measures ANOVA followed by Dunnett's *post hoc* analysis.

Figure 4.
Effects of chronic unpredictable stress (CUS) and riluzole on ^{13}C concentrations and -enrichment of Glu-C4, Gln-C4 and GABA-C2 after ^{13}C-acetate infusion. (**a**) Schematic depiction of the major metabolic pathways of ^{13}C isotopic label flow from the astroglial substrate, $[2-^{13}C]$acetate. After transport into the brain from blood, $[2-^{13}C]$acetate is metabolized in the mitochondria to acetyl-CoA (labeled at C2, Ac_2CoA) and enters the astroglial TCA cycle (TCA_a) as citrate labeled at C4 after condensation with oxaloacetate (oaa). Further metabolism along the cycle labels α-ketoglutarate (α-KG) at C4, which can then exchange with the astroglial glutamate pool (small pool glutamate) transferring ^{13}C label to C4 (Glu4). With time the ^{13}C label traverses the complete TCA cycle, labeling the other carbon positions, e.g., C3, C2, C1. The initial rate of label trapping at Gln4 is related to astroglial TCA cycle flux, whereas Glu_4 and $Gaba_2$ are related to the glutamate/GABA/glutamine cycle fluxes. Astroglia precursors (mainly Gln_4) are released from the astroglia, transferring the label to neurons for synthesis of Glu_4 and $Gaba_2$. Only flux pathways labeling Gln_4, Glu_4, $Gaba_2$ from $[2-^{13}C]$ acetate are shown. The pyruvate carboxylation pathway (anaplerosis) in astroglia is depicted by the dashed arrow. The continuous metabolism of unlabeled glucose in neurons and astroglia through pyruvate dehydrogenase (pyr $\rightarrow$ acetyl-CoA) serves as a constant dilution flux. Definitions: (subscripts) a, astroglia; n, neuron; acetyl-CoA, acetyl-coenzyme A; suc, succinate; TCA_n, neuronal TCA cycle. (**b**) ^{13}C concentration of Glu-C4 was significantly decreased by CUS ($F_{1,28} = 2.79$, P < 0.05) and this effect was reversed by riluzole treatment ($F_{1,28} = 7.8$, $P < 0.05$). (**c**) CUS significantly reduced ^{13}C concentration of Gln-C4 ($F_{1,28} = 13.71$, $P < 0.001$) and riluzole did not significantly reverse this effect. (**d**) ^{13}C concentration of GABA-C2 was significantly decreased by CUS ($F_{1,28} = 4.63$, $P < 0.05$) and this effect was reversed by riluzole treatment ($F_{1,28} = 5.39$, $P < 0.01$).

Figure 5.
Effects of chronic unpredictable stress (CUS) and riluzole on mRNA levels of glial-specific markers. (**a**) CUS showed a significant decrease of mRNA levels of glial fibrillary associated protein (GFAP) compared to home cage control (CTR) animals ($F_{1,16} = 4.52$, $P < 0.05$). CUS animals treated with riluzole were significantly different from CUS animals treated with saline and not different from CTR animals treated with saline or riluzole ($F_{1,16} = 5.7$, $P < 0.05$). Representative autograph of effect of CUS (**c**) on GFAP mRNA expression compared to CTR (**b**). (**d**) Although CUS has no effect on mRNA level of GLT-1, chronic riluzole treatment significantly increased GLT-1 transcript ($F_{1,16} = 4.9$, $P < 0.05$). Representative autograph of effect of CUS (**e**) on GLT-1 mRNA expression compared to a CUS animal treated with riluzole (**f**). Error bars represent s.e.m. *$P < 0.05$ compared to CTR and #$P < 0.05$ compared to CUS, two-way analysis of the variance (ANOVA), Student–Newman–Keuls post hoc analysis.

Table 1

Effects of CUS and riluzole on percentage of ^{13}C enrichment

Treatment	Glu-C4	Gln-C4	GABA-C2
CTR+Saline	4.0 ± 0.2	13.2 ± 0.2	2.6 ± 0.2
CTR+Riluzole	3.8 ± 0.1	12.8 ± 0.1	2.7 ± 0.1
CUS+Saline	3.4 ± 0.1[*]	11.5 ± 0.1[**]	2.0 ± 0.1[*]
CUS+Riluzole	4.0 ± 0.2[#]	12.6 ± 0.2[##]	2.8 ± 0.2[##]

Table summarizing the effect of CUS and riluzole on the percentage ^{13}C enrichment of Glu-C4, Gln-C4 and GABA-C2. CUS decreased the percentage of ^{13}C enrichment of Glu-C4 ($F_{1,28} = 3.4$, $P < 0.05$), Gln-C4 ($F_{1,28} = 12.12$, $P < 0.01$) and GABA-C2 ($F_{1,28} = 4.93$, $P < 0.05$).

Riluzole significantly reversed the effect of CUS on the percentage of ^{13}C enrichment of Glu-C4 ($F_{1,28} = 7.78$, $P < 0.05$), Gln-C4 ($F_{1,28} = 12.51$, $P < 0.01$) and GABA-C2 ($F_{1,28} = 7.696$, $P < 0.01$).

Error bars represent s.e.m.

[*]$P < 0.05$,

[**]$P < 0.01$ compared to CTR

[#]$P < 0.05$,

[##]$P < 0.01$ compared to CUS, two-way ANOVA, Student–Newman–Keuls *post hoc* analysis.

Table 2

Effects of CUS and riluzole on EAAT-1 and GS transcripts (% from CTR)

Genes	CTR + Saline	CTR + Riluzole	CUS + Saline	CUS + Riluzole
EAAT-1/GLAST	100 ± 7	101 ± 7	99 ± 11	98 ± 7
Glutamine synthetase	100 ± 7	100 ± 5	102 ± 5	104 ± 4